# Effects of acylurea insecticide in Manduca sexta larvae

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(The Independent, 1988)

"The outward form of the insect is fixed by the form of its external skeleton or cuticle. This cuticle is the product of a single layer of epidermal cells. Thus in the ultimate analysis it is the functional activity of the epidermal cell which is mainly responsible for the growth and form of the insect. The control of growth resolves itself into the control of the enzyme system contained within this cell."

Wigglesworth, V.B. (1959)

# Effects of acylurea insecticide in *Manduca sexta* larvae

Submitted by Darren Robert Chandler

for the degree of Ph.D. of the

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## Contents

## Abbrevations

| | |
|---|---|
| $\alpha$ | alpha |
| ANOVA | one way analysis of variance |
| $\beta$ | beta |
| Ci | Curie |
| cm | centimetre |
| cpm | counts per minute |
| dpm | disintegrations per minute |
| $\gamma$ | gamma |
| g | gram |
| h | hour |
| $IC_{50}$ | inhibitory concentration (50%) |
| ID | internal diameter |
| kDa | kilodaltons |
| kV | kilovolts |
| $LC_{50}$ | lethal concentration (50% mortality) |
| LD | light:dark |
| $LD_{50}$ | lethal dose (50% mortality) |
| M | molar |
| mA | milliamps |
| mCi | millicurie |
| $\mu$Ci | microcurie |
| mg | milligram |
| ml | millilitre |
| $\mu$l | microlitre |
| mm | millimetre |

| | |
|---|---|
| µm | micrometre |
| mmol | millimole |
| mM | millimolar |
| µM | micromolar |
| min | minutes |
| n | number of replicates |
| N | normal |
| ng | nanogram |
| nm | nanometre |
| $R_f$ | mobility relative to solvent front |
| SEM | standard error of the mean |
| w/v | weight by volume |
| U | enzyme activity units |
| v/v | volume by volume |
| x | times |

## Acknowledgements

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I would like to dedicate this to Helen for all her help and support, both moral and financial, throughout all the seemingly endless scientific dilemmas. My thanks to my family for their encouragement and to Kate Powell for my crash course in electron optics.

## Abstract

A variety of radiolabelled precursor and insecticidal studies were performed on the cellular physiology, cuticle formation and overall growth in larvae of the tobacco hornworm, *Manduca sexta*.

Fourth stadium larvae showed a dose-dependant response to the insecticide flufenoxuron and survivors failed to thrive in the next stadium if a successful moult was achieved. The development of an *in vitro* assay for flufenoxuron demonstrated its ability to inhibit chitin synthesis ($IC_{50}$ was 0.41 μM).

Some evidence was found of the effects of short-term exposure of flufenoxuron on proleg tissue explants at the electron microscope level. Epidermal rather than cuticular ultrastructural disruption was demonstrated.

The toxic effects of six acylurea insecticides showed a positive temperature correlation to insect mortality and sublethal growth inhibition. It is implied that temperature affects a process that occurs after insecticide uptake.

Flufenoxuron showed the ability to cause accumulation of the precursor to chitin and a radiolabelled analogue of the insecticide showed some selective binding to epidermal nuclear proteins.

Overall, the study shows the rapidity of acylurea action at the cellular level and the profound effect of temperatures likely to be experienced in the field upon its efficacy.

# Chapter 1

## Introduction

There are various means available to Man to disrupt insect pest populations. These include chemical control using a variety of insecticides and insect growth-disrupting chemicals; microbial control using bacterial, viral and fungal agents and biological control using natural insect predators, parasitoids and genetic sterilization programmes. Of these, chemical control is today by far the most widely used.

The chemical tools for insect control have been heavily exploited over the last fifty years with the raw screening products coming mainly from the petroleum industry. Indeed it has become more difficult to identify a novel insecticidal compound. Statistics show over 12 000 compounds had to be screened in 1977 to identify one active compound, whereas in 1956 the figure was only 1800 (Kumar, 1984). Ideally such a chemical should possess certain properties under the conditions for its intended use. Essentially it should stay at the place of application through its active period, being toxic to particular pests but harmless to other organisms including Man. The chemical must persist long enough in the environment to be effective, but nevertheless break down into harmless products within a reasonable time and must also be inexpensive to manufacture.

The discovery in 1939 by Paul Müller of the insecticidal properties of 1,1,1-trichloro-2,2-bis (p-chlorophenyl)-ethane (DDT) was rapidly followed by its widespread use in both medical and agricultural insect control. As is well known,

this ultimately led to serious and damaging effects on the environment, highlighted in public concern by Rachel Carson's 'Silent Spring' (1962). Actually these consequences had not been entirely unforeseen, and Wigglesworth had warned of the likely ecological effects of DDT as early as 1945. DDT was banned in the USA in 1972, and its use worldwide has now been severely limited. The story of DDT is summarised by Mellanby (1989).

The problem with DDT is not its intrinsic toxicity to non-target organisms (actually DDT is acutely one of the safest insecticidal chemicals), but its extreme resistance to breakdown and consequent persistence in the environment. This allows its accumulation in the food chain to very high levels, with deleterious effects on top predators.

DDT's persistence would in any case in the long-run have caused severe problems with resistance in target insects. DDT-resistant houseflies were observed as early as 1946 (Mellanby, 1989) and large numbers of instances of DDT resistance in disease vectors were catalogued by Georghiou and Taylor (1976).

As much as any other factor, it is the continuing development of resistance to existing insecticides that drives the search for new ones. The development of synthetic insecticides progresses hand in hand with knowledge of insect biochemistry as it is unravelled. This was pointed out by Sir Vincent Wigglesworth (1956) who said " If we had sufficient knowledge of insect biochemistry, it should have been possible to predict the activity of.... particular substances; and....to devise molecules which will disrupt the machine at whatever point we desire." However in practice, as he pointed out, the situation is the reverse, with newly discovered insecticides shedding light on basic insect biochemistry.

In the search for novel insecticides, selectivity against insect targets is an important goal. Clearly it is an attractive attribute in any new group of insecticidal

chemicals that they attack a biochemical or physiological target present only in the targets, and not in other organisms. A group of insecticides which almost fulfills this criterion is the acylureas, which are known to interfere with cuticle deposition in insects. These chemicals are apparently harmless to non-Arthropods, and in particular do not affect Vertebrates. These recent additions to the insecticide armoury are further discussed in this chapter in terms of cuticle structure, formation and disruption at both the physiological and biochemical levels.

## General Insect Cuticular Structure

### *Insects and the Cuticle*

The insect integumental cuticle is of vital importance in the insect's survival; acting as a supporting structure (exoskeleton), waterproofing, housing sensory receptors and a site for muscle attachment (Neville, 1975). As a consequence of the cuticle's exoskeletal properties (stiffness and rigidity) an insect must form new cuticles and shed (ecdyse) old confining ones in order to grow. The moulting from one larval stage (instar) to the next also permits changes in the skeletal form of the cuticle.

### *Cuticle Fine Structure*

Cuticle is an extracellular, multilayered material secreted by the underlying epidermis. It consists of a thin, outer epicuticle and an inner procuticle which is much thicker. The outer layers of the procuticle consist of pre-ecdysial exocuticle (which may be tanned) and its inner layers form the post-ecdysial endocuticle (usually tanned). A layer immediately next to the epidermis is called subcuticle and is thought to be cuticle that has yet to stabilised into the endocuticular form. The single layer epidermis produces all the components for the exoskeleton and this, through time, can serve as a permanent record of

secretory events. A schematic diagram showing the appearance of integumental cuticle in cross-section is given in Fig. 1.1.a.

*Epicuticle*

The epicuticle is the thin external coating covering nearly all the insect body surface area as well as the fore- and hindgut. Certain exceptions include chemoreceptors and the midgut. The epicuticle is the non-chitinous portion of the exoskeleton and is composed of 4 layers (Fig. 1.1.b.). Starting with the innermost layer, using Weis-Fogh (1970) terminology they are known as the inner and outer epicuticle, the wax layer and finally the cement layer. The inner and outer epicuticles are secreted before ecdysis (moulting) while deposition of the cement and wax layers follows ecdysis.

*Cement Layer*

This is secreted post-ecdysis most probably by the dermal glands in the epidermis via ducts. It is thought to consist of tanned protein and lipids (Beament, 1955) acting as a buffer to abrasion. Sometimes the epicuticle is only a trilaminar structure (Locke, 1969). In Apis, the honey bee, the waxlayer is the outermost layer, the cement layer being completely absent (Locke, 1961).

*Wax Layer*

The lipids in this layer may, on the whole, permeate in-to the overlying cement layer (Locke, 1974) whilst there are major arguments against the existence of a discrete monolayer as proposed by Beament (1959). These arguments are briefly summarized by Hadley (1984). The chemistry of the lipids shows them to be long chain saturated alcohols esterified with fatty acids. The labile nature of these waxes has hindered the study of this layer. Physiologically they are important in reducing water permeability (Beament, 1959) because damage to the epicuticle by abrasive materials leads to water loss. Severe wounds to the

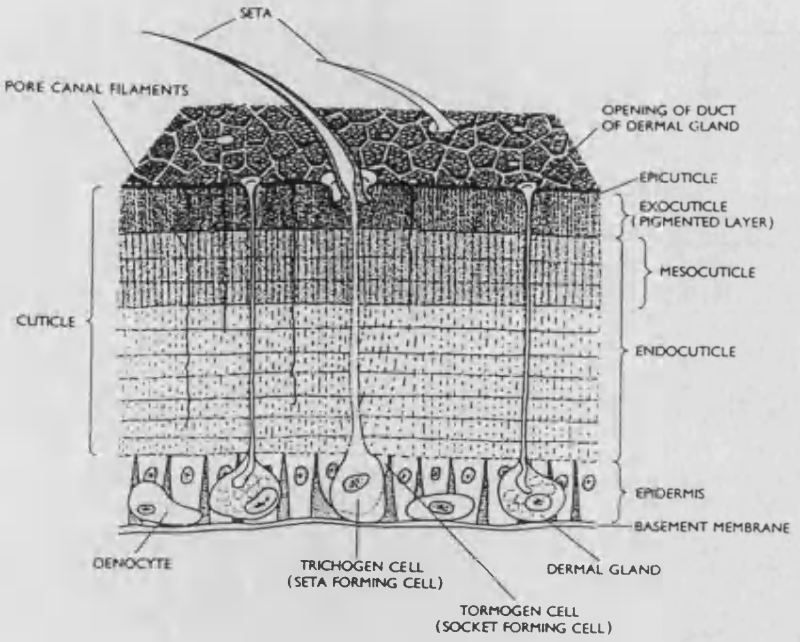



**Fig. 1.1.a.** Generalised diagram of the structure of the insect integument (from Filshie, 1980).

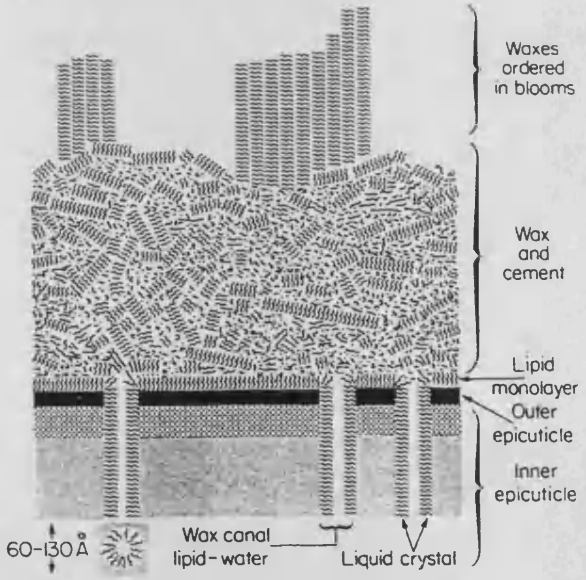



**Fig. 1.1.b.** Schematic diagram of an insect epicuticle. In this instance the most complex situation is illustrated. Thus, wax is associated with the cement layer and wax also occurs in a bloom above the cement layer (from Hepburn, 1985).

integument become plugged with dark amber coloured material called sclerotin (Pryor, 1940; formerly referred to as cuticulin by Wigglesworth, 1937) which is stabilized lipid.

*Outer Epicuticle*

Sometimes called cuticulin (Locke, 1966) and not to be confused with sclerotin, this is the first layer of the newly forming cuticle secreted from the apical brush border plaques of the epidermal cells. In appearance it is a distinct layer up to 18 nm thick (Locke, 1966). Locke's term 'cuticulin' is somewhat confusing because earlier Wigglesworth (1933) had described cuticulin as a substance traversing both the inner and outer epicuticle. Shortly after the layer is formed, 3 nm pores can be observed within this layer (Locke, 1961). During the development of the pharate cuticle the old cuticle is enzymatically digested by moulting fluid enzymes present in the moulting fluid. Since the products of digestion are recycled incorporated into the new cuticle (Gwinn and Stevenson, 1973a), it may be that this recycling takes place through these pores. Since the new cuticle is not digested by the moulting fluid the outer epicuticle of this new cuticle must be resistant to enzymatic degradation.

*Inner Epicuticle*

The inner epicuticle or dense layer (Locke, 1966) is about 0.5-2.0 μm thick and is secreted after the formation of the cuticulin layer. This layer is produced by secretory vesicles from the Golgi complex within the epidermal cells. Extracellular enzymes present in this layer are responsible for the repair of superficially scratched cuticle by hardening (tanning) the wound. These phenoloxidase enzymes oxidise diphenols to quinones, which in turn cross-link with the cuticular proteins. These enzymes can be inhibited, and therefore wound darkening prevented, with phenylthiourea (Dennell, 1958) and

diethyldithiocarbamate, both of which are known to inhibit copper containing oxidases.

*Procuticle*

The procuticle, is by definition, the rest of the cuticle. It contains protein and rods of the polysaccharide chitin. It comprises exo- and endocuticle. Exocuticle is laid down prior to ecdysis, while endocuticle is deposited afterwards. In some cases the exocuticle is sclerotised (chemically stabilised), but sometimes it is not. There are regional differences within an insect (for example intersegmental membranes are never hardened), and also differences according to stage. Soft-bodied larvae for example do not for the most part sclerotise ('tan') the exocuticle. It should be noted that in some cases (e.g. adult locusts) the endocuticle is sclerotised in addition to the exocuticle. For tanning, tyrosine derivatives form cross-links within the cuticle giving added stability in addition to the extracellular enzymes mentioned above. The chemistry of tanning, a complex and poorly understood process, is reviewed by Kramer *et al.* (1987), Sugumaran (1988) and Andersen (1989).

*Exocuticle*

This is often heavily tanned, when as a result it cannot be recycled at a moult by enzymatic hydrolysis. When viewed in cross-section the arrangement of the chitin rods gives the appearance of arcs within the cuticle. Bouligand (1965) realised that this appearance of arcs is the consequence of an underlying helicoidal structure, the unidirectional chitin fibres in a component layer being orientated a few degrees counter-clockwise to the overlying layer and so the arcs are mere artefacts. When the consecutive layers have rotated through $180^{o}$, one set of arcs is generated and is called a lamella (Fig. 1.2.).

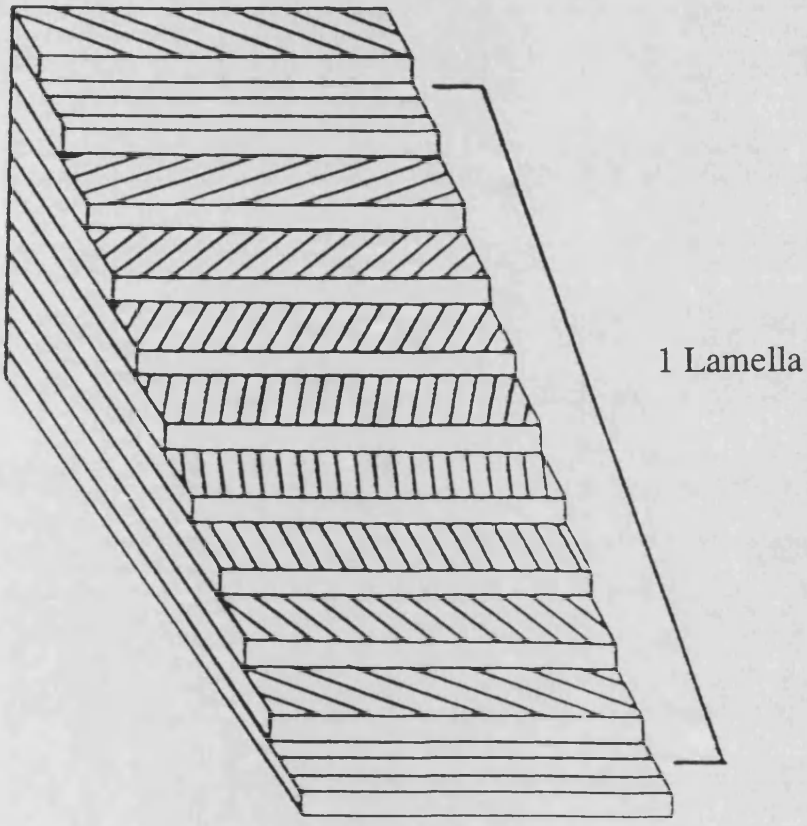



**Fig. 1.2.** Diagram of helicoidal structure. In each component layer chitin crystallites are arranged in parallel. The direction of each successive layer changes progressively in the direction of a left-handed corkscrew. An oblique section of such a system (shown here by drawing a wedge-shaped stack) then exposes the crystallites, forming a pattern of concentrically nesting arcs. Each half rotation of $180^{o}$ generates one set of arcs as shown (from Neville, 1984).

*Endocuticle*

The endocuticle appears as thick layers of uni-directional chitin fibres interspersed with thin helicoidal regions in winged adult insects. In larvae, the endocuticle is helicoidal throughout. This can be digested by the moulting fluid enzymes and resorbed as it is untanned. Daily growth layers are laid down here, giving an account of cuticular deposition occurring by day (non-helicoidal) and by night (helicoidal). This circadian rhythm means that the exact age of an insect can be determined. This has been the subject of a review by Neville (1983).

*Subcuticle*

Here, the architecture of the cuticle is arranged to form either the exo- or endocuticle. It is a continual process of stabilisation and organising newly secreted components from the cuticle. It is also known as the deposition zone.

*Epidermis*

The epidermis is a monolayer producing cuticle of the integument and also the structures associated with the cuticle e.g. tracheae and sensory hairs. The activity of the epidermal cell varies during the moult cycle of a larval instar with the cell taking its cues for actions such as changing cellular shape (Wielgus and Gilbert, 1978) or dividing, from either the haemolymph (general factors) or the adjacent cells (local factors) (Wigglesworth, 1940).

In appearance, an epidermal cell contains the common organelle structures (Fig. 1.3.). The apical membrane surface has a vital role in the larval moult cycle because cuticular components synthesized within the cell are released here. Additionally, chitin microfibres appear to be synthesised at this membrane surface. This process is performed by the microvilli tipped with plasma membrane plaques (PMP) on the apical surface. The plaques appear to be involved in the polymerization of chitin precursor into the final rod crystallite. However it is not

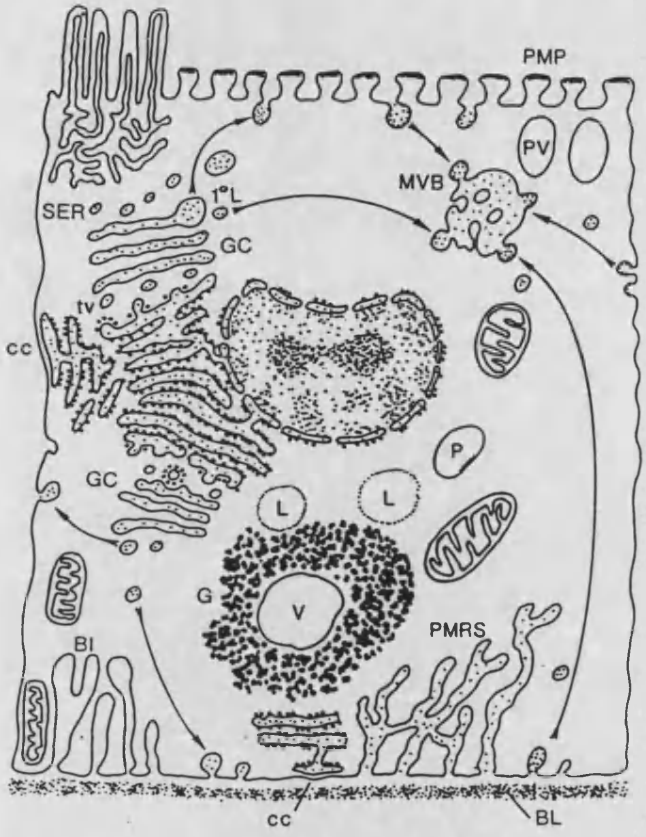



**Fig. 1.3.** The main features of an epidermal cell. *BI* basal infold; *BL* basal lamina; *cc* confronting cisterna; *G* glycogen; *GC* Golgi complex; *L* lipid droplet; *MVB* multi-vesicular body; *P* peroxisome; *1° L* primary lysosome; *PMP* plasma membrane plaques; *PMRS* plasma membrane reticular system; *PV* pigment vacuole; *SER* smooth endoplasmic reticulum; *tv* transition vesicles of GC; *V* vacoule often associated with glycogen (from Locke, 1984).

clear whether the plasma membrane plaques are themselves capable of organising cuticular assembly or are merely responsible for the passage of precursors through the membrane to a site of assembly (Locke, 1976). In production of cuticle for the next larval instar, patches of cuticulin appear directly above the plaques in the pharate larva, fusing together as they increase in area until the surface is completely covered. The close association of the cuticle with the cell in this fashion means it is possible for the cell to influence the properties of the cuticle. It can alter the extendibility of the cuticle which is especially important in blood sucking (haematophagous) insects such as the mosquito and the cone-nosed bug (*Rhodnius prolixus*). The reasoning behind this is outlined later on. At apolysis there is a rapid mitotic division and folding of the epidermis and this results in synthesized cuticle having a larger surface area than the old cuticle. This permits the larva to grow larger during the following instar.

*Cuticular Properties*

Cuticle has similar properties to the fibre composite fibre glass. Each new layer is laid down a few degrees anti-clockwise (helicoidal) to the previous layer. This prevents crack formation but at the same time provides required strength without cumbersome weight and helps to assert insects as the undisputed, successful animal class. Cuticle is a composite where fibres made from chitin are associated with a protein matrix. These fibres have been reported to have a mean diameter of 2.8 nm (Neville *et al.*, 1976) and up to a few μm long. Extensive inter and intramolecular hydrogen bonding explain the high stability of chitin making it, by definition, insoluble in hot dilute alkali. Neville *et al.* (1976) using optical diffraction calculated a microfibril to contain, on average, 3 sheets of 6 chains of chitin (N-acetylglucosamine residues). This arrangement is outlined in Fig. 1.4.a.

*Forms of Chitin*

Chitin occurs not only in insects but also forms the cell walls of most yeast and filamentous fungi. In insects it is distributed mainly in the procuticle but also lines the fore- and hindgut peritrophic membranes, trachae and tracheoles. It is a polysaccharide composed of 1-4 β-linked N-acetylglucosamine (GlcNAc) monomers (Fig. 1.4.b.) having a molecular mass of 1-2 x $10^3$ kdal. This approximates to 5-10,000 GlcNAc units in a chain length of chitin. The overall biochemistry and physiology of insect chitin is reviewed by Kramer and Koga (1986). The crystalline forms of chitin have been extensively studied by Rudall and Kenchington (1973) using X-ray diffraction technology. There are 3 known polymorphs of chitin, these being α, β and γ chitin. The forms correspond to the arrangement of the chitin chains in successive sheets within the microfibril (Fig. 1.4.c.) and each form though variable in degree of crystallinity, is classified as a viscoelastic polymer (Hepburn and Chandler, 1980).

*α-Chitin*

α-chitin is the most abundant and stable chitin type, occuring in arthropod exoskeletal cuticle and fungi (Muzzarelli, 1977). The α-chitin structure, as proposed by Carlström (1962), consists of neighbouring chitin sheets having their respective β 1-4 linkages arranged in opposite directions. Thus successive sheets are said to be anti-parallel.

*β-Chitin*

β-chitin, in which the chains are all arranged in parallel, is the least common form. Initially recognised in the pen of the squid *Loligo* (Lotmar and Picken, 1950) its x-ray diffraction pattern was interpreted by Blackwell (1969) in diatom spines. In arthropods it is known to occur only in the cocoons of the figwort weevils *Cionus spp.* and *Cleopus pulchellus* and the cerylonid beetle

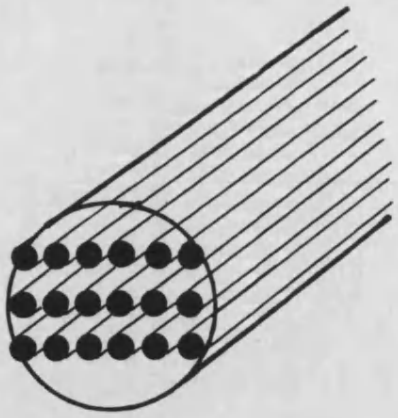


**Fig. 1.4.a.** Common structure of a chitin microfibril depicting a 3x6 chain arrangement.

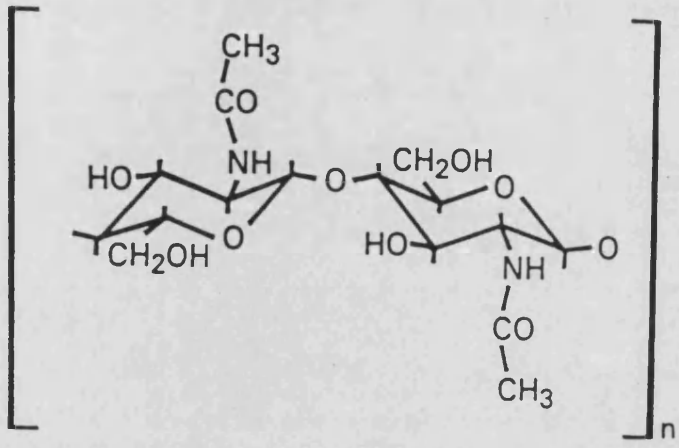



**Fig. 1.4.b.** Generalised chemical structure of chitin composed of repeating monomers of 1-4 β-linked N-acetylglucosamine.

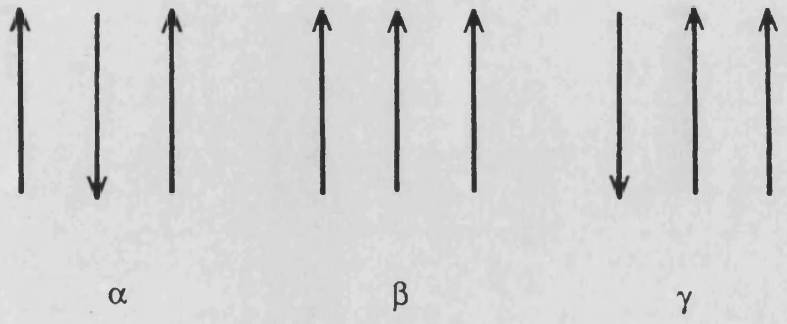



**Fig. 1.4.c.** The three known polymorphs of chitin chain arrangement.

*Murmidius ovalis* (Rudall and Kenchington, 1973). These cocoons are constructed from microfibrous "ribbons" of β-chitin secreted in the midgut.

*γ-Chitin.*

In γ-chitin chain directions in successive sheets adopt a 2 up, 1 down orientation. This polymorph, as suggested by Rudall (1963) was also discovered in *Loligo*, in the stomach cuticle (Rudall, 1962). It is found in peritrophic membranes of several insect species (Rudall and Kenchington, 1973) as well as other weevil cocoons (Kenchington, 1976).

*Cuticular Proteins*

Proteins are the major constituent of the procuticle, forming the matrix, but very little is understood about the proteins themselves and their interaction with the chitin microfibrils. Electron micrographs of cross-sections of cuticles, sectioned across the microfibrils, show an array of unstained centres surrounded by electron-dense material (Rudall, 1967). This pattern has been interpreted as micrifibrils of chitin in a protein matrix. In the light of what is now known the pattern would be more correctly interpreted as chitin microfibrils surrounded by and bound, by covalent hydrogen bonds, to protein chains (Hackman, 1976). This complex is immersed in a matrix of loosely bound protein (Fig. 1.5.).

Only recently has work started to sequence the proteins (Willis, 1989) although it is possible to extract and separate whole proteins into bands by 2-D electrophoresis. Proteins can be removed by treating the cuticle with hot, dilute alkali the remaining material being deemed chitin. The amino acid composition of the proteins within tanned cuticles is quite different to that of proteins found in the untanned regions, the latter having significantly higher amounts of polar amino acids (Hackman, 1984). It may be that the different properties required by the cuticle for different anatomical regions may be achieved in part by varying the

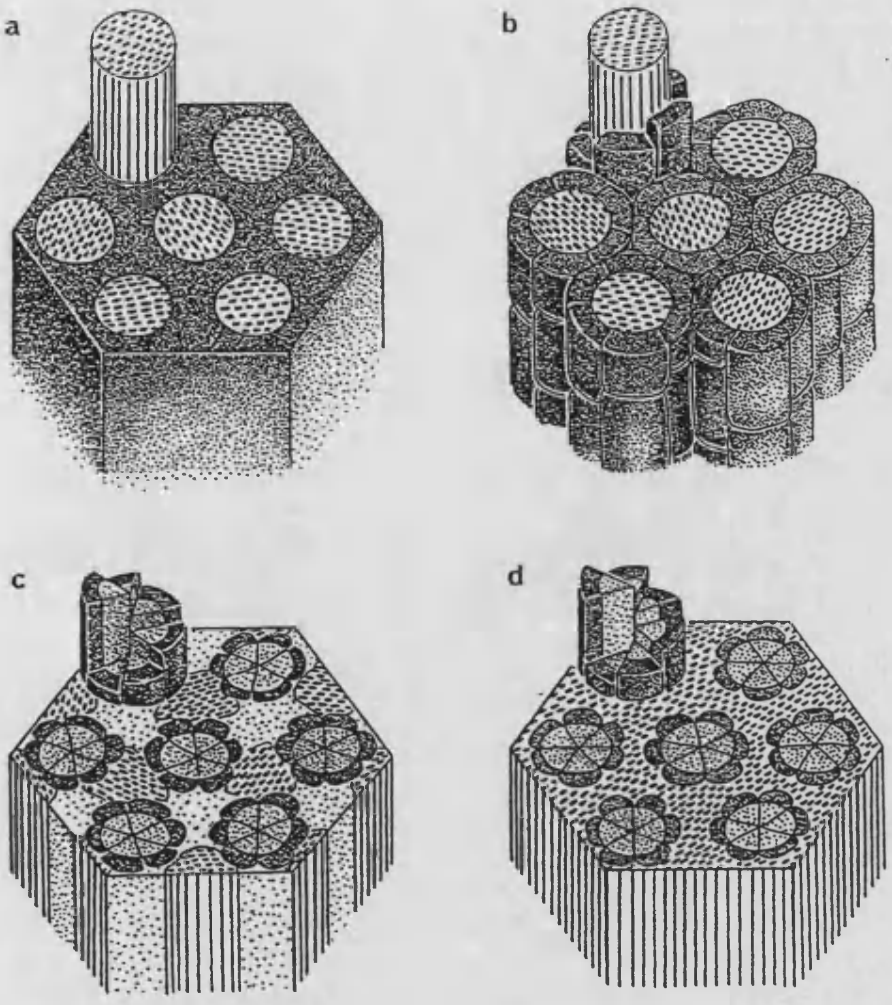



**Fig. 1.5.** Different cuticle models. a) Parallel chitin crystallites cemented with proteins; b) Parallel chitin crystallites surrounded by helically arranged proteins; c) polar and non-polar parts of proteins segregated; clear rods are supposed to be formed by polar amino acids and electron dense alveolar walls are supposed to contain polar amino acids and separated chitin crystallites; d) model slightly different from that proposed in c. The chitin lattice is connected amd more or less regularly ordered (from Giraud-Guille and Bouligand, 1986).

protein:chitin ratio but also varying the proteins themselves (Skelly and Howells, 1988). As already mentioned, cuticular extendibility is important in the growth of a larva throughout an instar (due to the cuticle being folded at the start of the instar) but more so in haematophagous insects. In *Rhodnius prolixus*, the cuticle proteins are adapted making the modified cuticle capable of stretching during the course of feeding on a blood meal (Hackman, 1975).

*Cuticular Lipids*

Although not the major constituent of the insect cuticle the lipids are vital in the prevention of dehydration especially considering the large surface area/volume ratio. The waterproofing of the cuticle occurs in the wax layer of the epicuticle, the synthesized lipids passing through the epidermis to the epicuticle probably via the pore canals as described by Locke (1961). Lipids may act as structural components in the epicuticle. These subsequently become tanned forming a substance first described by Wigglesworth ("cuticulin", Wigglesworth, 1933). These components are reviewed by Lockey (1988).

In addition to a water regulatory role, cuticular lipids provide protection from micro-organisms and control the entry of insecticides. Koidsumi (1957) reported that the cuticular lipids of the larvae of *Bombyx mori* L. and *Chilo simplex* Butler were capable of restricting the growth of pathogenic fungi *in vitro* again highlighting the exoskeleton as a physical barrier to the environment.

## Chitin Synthesis

The pathway from precursor to final product is best known from studies in fungi but it is thought that the pathways operating are the same for insects (Kramer and Koga, 1986). There is a cascade of transformations converting

glucose into the chitin polymer. The synthesis of precursors takes place in the epidermal cells underlying the cuticle (Surholt, 1975; Vardanis, 1976). The Hexosamine Pathway (Fig. 1.6.) which outlines chitin synthesis, was proposed by Candy and Kilby (1962) upon showing the presence of enzymes necessary for chitin formation in a *Schistocerca gregaria* wing homogenate, converting trehalose or glucose to uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), the final precursor to chitin. However, they failed to show the polymerization step into chitin whereby UDP-GlcNAc is transported through the cell and on interacting with the enzyme, chitin synthase, splits the substrate (UDP-GlcNAc) into GlcNAc which attaches to the growing chitin chain and UDP which re-enters the cell pool. The pathway from UDP-GlcNAc to chitin in a cell-free preparation was finally determined by Jaworski *et al.* (1963) using extracts from *Prodenia eridania* but the enzyme is unstable *in vitro*.

All the steps up to the formation of UDP-GlcNAc are catalyzed by cytoplasmic soluble enzymes. Chitin polymerization is thought to be catalyzed by a membrane bound enzyme, chitin synthase (CS) [UDP-2-acetamido-2-deoxy-D-glucose: Chitin 4-acetamidodeoxyglucosyltransferase, EC 2.4.1.16], with the active site exposed on the external surface of the epidermal cell (Mitsui *et al.*, 1984) with access only via the cytoplasmic surface of the plasma membrane plaque. The major practical problem encountered in studying insect CS is in the isolation of the enzyme as a cell-free extract which would then allow direct insecticide inhibitor studies (Fristrom, 1968; Surholt, 1975; Vardanis, 1976; Retnakaran and Hackman, 1985), a problem not encountered with fungal and yeast CS (Glaser and Brown, 1957; Muzzarelli, 1977; Ruiz-Herrera *et al.*, 1977; Duran and Cabib, 1978). Only recently has some headway been made in improving cell-free insect synthesizing systems even if there is only partial enzyme recovery. The breakthrough was initially made by Cohen and Casida (1980a) and Mayer *et al.* (1980) purifying gut CS from larvae of *Tribolium* spp

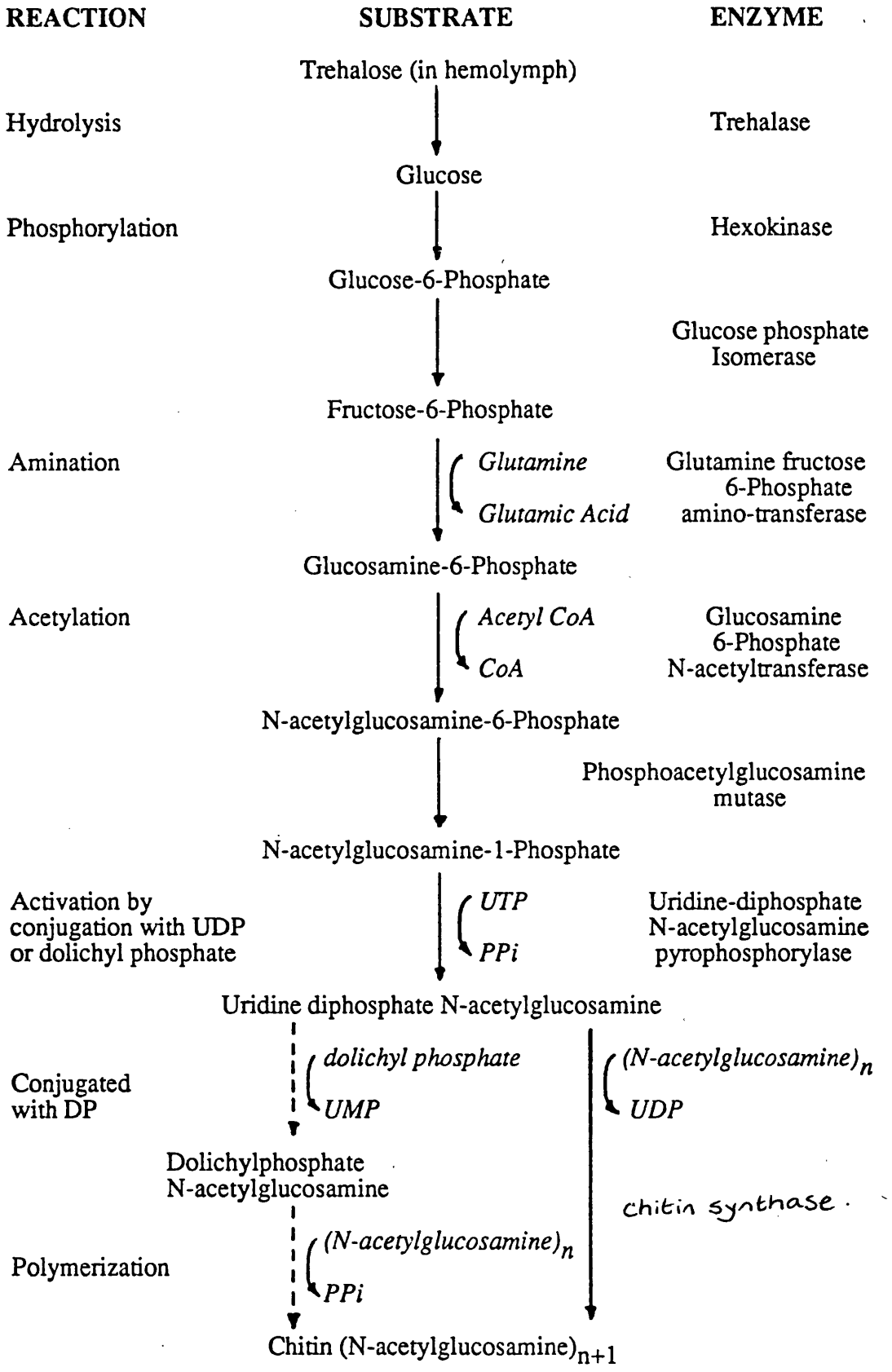



**Fig. 1.6.** The Hexosamine Pathway (from Kramer *et al.*, 1985).

and *Stomoxys calcitrans* respectively. Further work led to integumental CS isolation (Cohen and Casida, 1982; Turnbull and Howells, 1983). Using isolated whole integument has also made it possible for mass screening of potential Insect Growth Regulators (IGR's) (Hajjar and Casida, 1978; Turnbull and Howells, 1982; Kitahara *et al.*, 1983) although any disturbance of the epidermal cells is detrimental to normal chitin synthesis (Surholt, 1975).

Another recent advance has been the development of insect cell lines capable of producing chitin (see Marks and Ward, 1987) but whether this is cuticular α-chitin has yet to be established. Many such cell-lines have now been developed to allow further IGR investigation to an extent where crystals of an IGR have been visualised within the cells at high doses (Klitschka *et al.*, 1987).

*Site of Synthesis*

The presumed CS units are found grouped together (Locke and Huie, 1979) at the microvillar surface of the plasma cell membrane (Vardanis, 1979; Horst, 1981) where the catalytic site of the enzyme is on the outside of the cell membranes (Fig. 1.7.). Work carried out by Mitsui *et al.* (1984) using a preparation from mid-gut membranes of *Mamestra brassicae* led them to postulate that there must be a transport protein present within the membrane to transfer UDP-GlcNAc to the CS units. This is a different transport protein to the lipid carrier dolichyl phosphate which binds the substrate (UDP-GlcNAc) forming dolichyldiphospho-GlcNAc which then polymerises directly with the chitin chain (Kramer *et al.*, 1985). The location of the enzyme within the apical membrane is still unknown (Chen and Mayer, 1985). The difficulty found in purifying enough CS material combined with its instability has meant to date that the subunit structure of the enzyme is also unknown. At present it is assumed that the insect CS is analogous to the yeast and fungal CS.

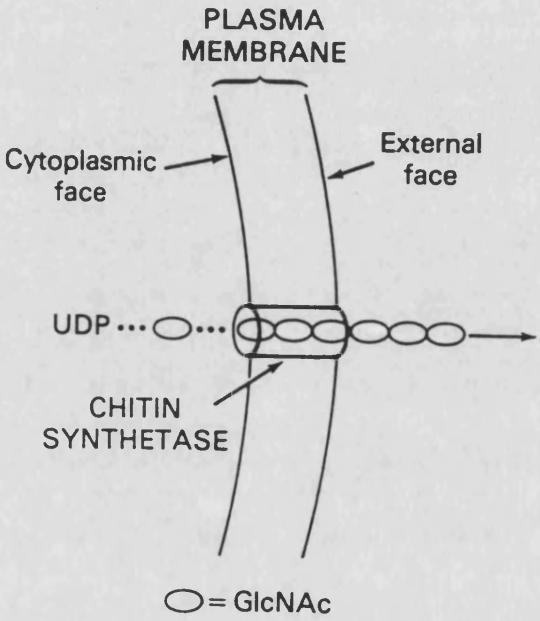



**Fig. 1.7.** Scheme of vectorial synthesis of chitin through the plasma membrane. Chitin synthase is depicted as spanning the membrane (from Cabib, 1987).

## Moulting

The process of moulting is necessary, as already mentioned, for further growth and development in arthropods as a result of the relative rigidity of the exoskeleton. This process is hormonally controlled and always follows a non-moulting phase (Fig. 1.8.). It is this basic need to synthesise new cuticle and shed old, confining ones which offers a potential source for insect control through the use of specific insecticides interacting within this process.

The initiation of the moult is probably due to proprioceptors in the exoskeleton although none have been identified to date. This is more apparent when one considers that the hemipteran *Oncopeltus fasciatus* (Nijhout, 1979) and the lepidopteran *Manduca sexta* (Truman, 1978) must both obtain an "optimal" weight to moult to the next instar. Once critical weight has been reached it is possible to moult through the "gating" of the release of PTTH (Prothoracicotropic hormone) from the brain. If the critical weight for moulting is not attained by a certain time of day, the insect will have to wait another 24 hr before the next occurrence of the critical time in the light/dark cycle. This physiologically older insect will then become a Gate 2 larva (Truman, 1972) whereas larvae attaining this specific weight 24 hr earlier for moulting are Gate 1 larvae. In 4th instar *Manduca sexta* this is typically 0.8 g.

### *Moulting-related events*

The onset of moulting is marked by mitotic activity in the epidermis followed by an increase in the size of the epidermal cells (Zacharuk, 1972) and is initiated by a rise in ecdysteroid titre due to PTTH stimulation. This is followed by apolysis (Jenkin and Hinton, 1966) which is the separation of the epidermal cells from the cuticle. However, it is not clear whether this separation is due to retraction of the epidermal cells or secretion of material between the plasma

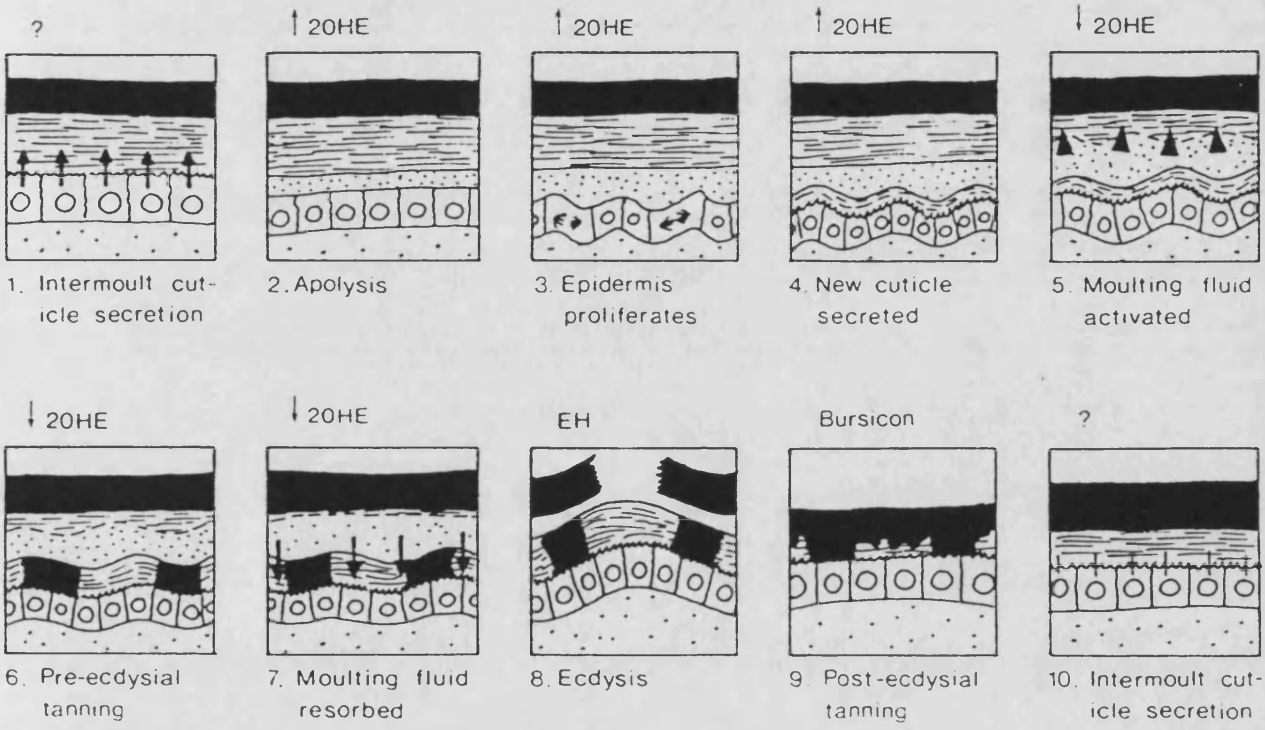



**Fig. 1.8.** The moulting cycle: Individual panels represent cellular activities. The diagrams attempt to show a generalised moult cycle. In some insects some stages may be absent. For example, soft-bodied larvae do not undergo bulk tanning of the pre-ecdysial cuticle in most parts of the integument (from Reynolds, 1987).

membrane and the old cuticle. Zacharuk (1976) recognised this physical separation to be a separate event to the enzymolysis of the old endocuticle.

The exuvial space that is thus formed is separated from the epidermal cells by the latter's secretion of an exuvial, or moulting membrane (Passonneau and Williams, 1953) which acts as an impervious cuticulin layer to any lytic enzymes due to pre-ecdysial tanning. In some flies (Filshie, 1970) and beetles (Zacharuk, 1972) no such membranes are seen making the supposed protective role of the layer an enigma.

Moulting fluid, secreted into the exuvial space, contains the enzymes necessary for degradation of the old endocuticle. The fluid, once activated, attacks only the endocuticle with chitinases and peptidases that digest both chitin and protein. The products of cuticular digestion are largely reabsorbed by the insect before ecdysis either through the general body surface (Wigglesworth, 1974) via the 3 nm pores or the digest is drunk by the insect at ecdysis (Zacharuk, 1972). By the process of recycling cuticular digests Gwinn and Stevenson (1973a) found over half of radiolabelled cuticular products from old endocuticle in the cuticle of the new larva. The hormonal control of moulting fluid activation or of the latter reabsorption has not been studied in detail but it seems likely that the decline in ecdysteroid below some threshold level is responsible for triggering the reabsorption and possibly also for activation (Riddiford, 1985).

The continued deposition of cuticle of the pharate (Hinton, 1946) larva (a larva within the confines of the old larval cuticle) and reabsorption of the moulting fluid/endocuticular digest is followed by ecdysis itself. At least in Lepidoptera, this is additionally dependant on the release of another hormone, the neuropeptide eclosion hormone (Truman, 1985).

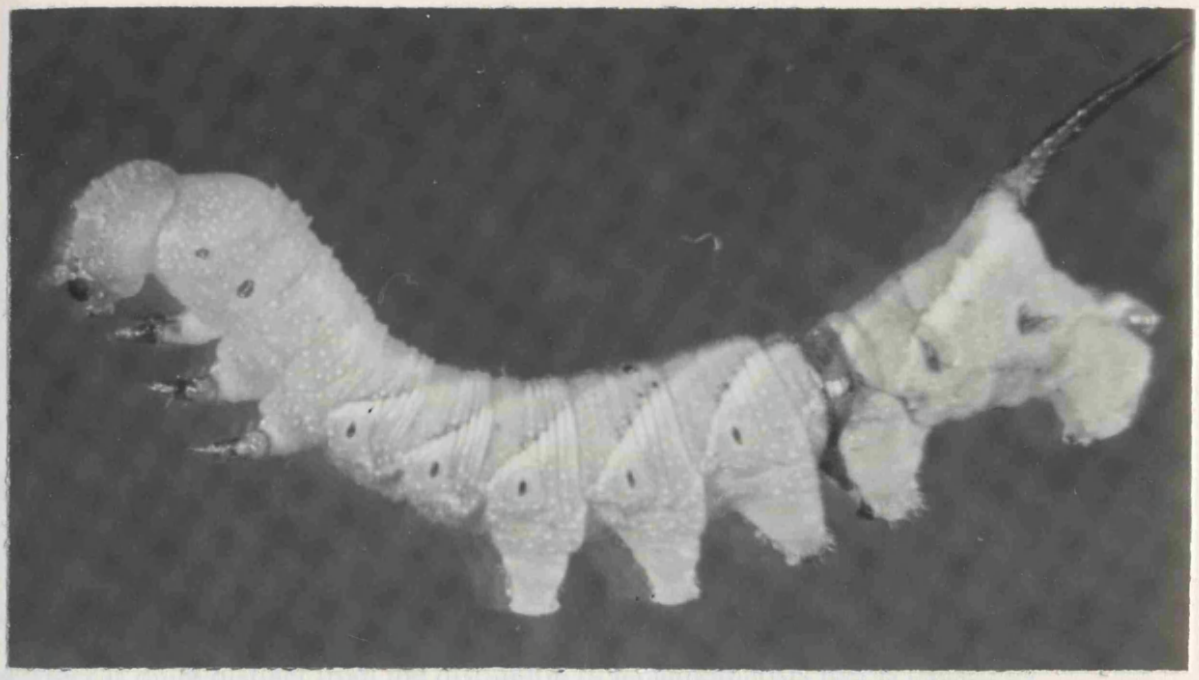
At ecdysis , the cuticle readily splits at the ecdysial line or Häutungsnaht (Wigglesworth, 1974) which is a T-shaped line of weakness on the head and thorax. The larva contracts the abdomen forcing hemolymph into the head and thorax. This increase in internal pressure causes the old cuticle to split so releasing the new larval instar. Morphogenic processes at the moult (i.e. whether or not morphogenesis occurs) are governed by yet another group of hormones, the juvenile hormones. These determine by their presence or absence the progress of a larval insect to another larval stage, or to a pupa, and likewise pupa to adult. Juvenile hormone titres are also concerned with regulation of ecdysteroid titres, and thus the decision to moult. It is beyond the scope of this chapter to consider juvenile hormones in any detail, but it is worth noting that chemical interference with juvenile hormone function in the form of synthetic juvenoids (juvenile hormone analogs) has proved to be an effective way of controlling at least certain groups of insects (a recent review of this is given by Menn *et al.*, 1989).

## Cuticular Disrupting Insecticides

### *Insect Growth Regulators*

The vulnerability of the insect cuticle is associated with the need for the insect to moult when the old cuticle becomes too small thus restricting growth. Chemical compounds which interfere with chitin deposition into the thickening cuticle, especially between apolysis and ecdysis, offer possibilities as insecticides especially considering their low mammalian toxicity (see reviews by Chen & Mayer, 1985; Hajjar, 1985). These compounds act as 'Insect Growth Regulators' (IGR's) in that they cause delayed mortality which is dependant on growth. The term IGR was originally used for juvenoids, but it is now often used to include chitin synthesis inhibitors. These later inhibit the formation of new cuticle to replace that which is to be shed. The insects start to moult as normal but they fail

a



b

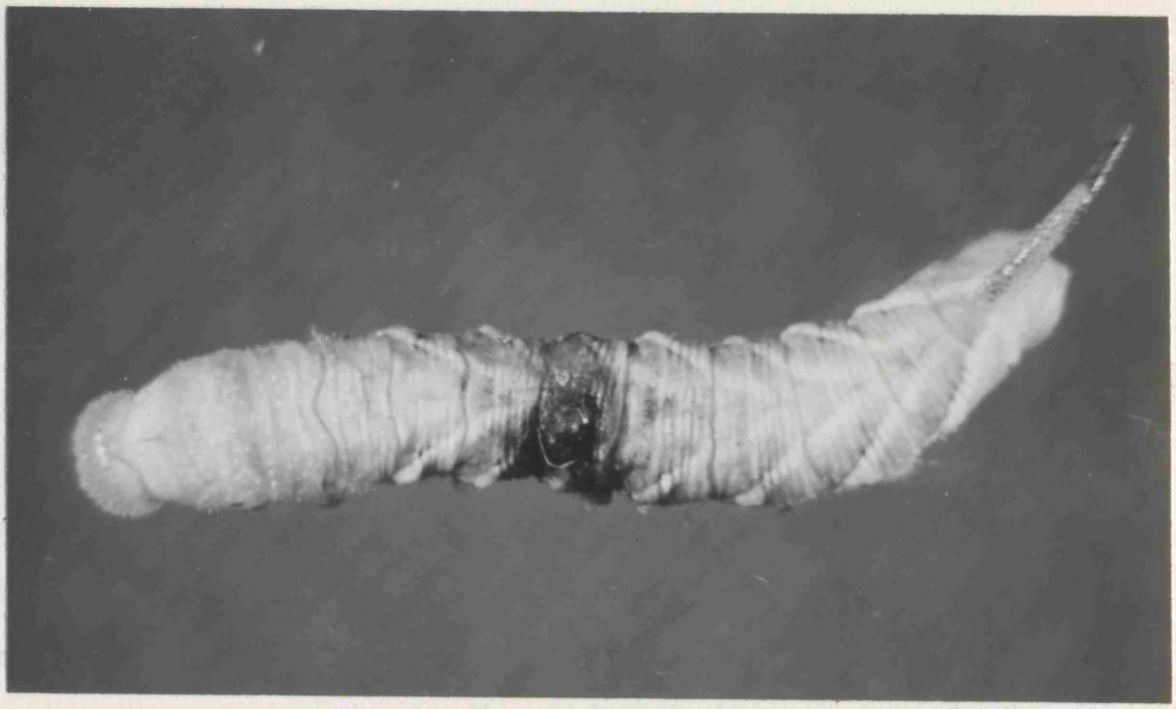


**Fig. 1.9.** Photographs of day 3 fourth stadium *Manduca sexta* larvae after an unsuccessful moult due to sublethal poisoning from flufenoxuron. **a**) Note the larva enclosed within the old third stadium exuvia which has not been properly shed). **b**) The larva has burst whilst attempting to moult - unsuccessfully.

to shed the old cuticle properly (Fig. 1.9.). The old cuticle then dries out and contracts, thus constricting the posterior of the larva. With the new cuticle being insufficiently strong to maintain the hydrostatic pressure for body form they may simply burst at a weak point on the new cuticle (normally the intersegmental membrane). Sometimes they may fully ecdyse but fail to reinitiate feeding (Guyer & Neumann, 1988) which may be due to morphologically disrupted jaws (Retnakaran *et al.*, 1985). Sometimes, inhibitors of chitin synthesis disrupted metamorphosis; the moult between the last instar to pupa is completed but the new pupa is malformed and the adult insect fails to emerge. The mechanism of this effect is quite distinct from the disruption of metamorphosis that is caused by juvenoid IGR's. Nevertheless, its end result is the same.

Juvenile Hormone (JH) analogues and anti-JH are synthetic compounds used to disrupt larval-larval moults. As already mentioned, the presence of JH ensures a larval-larval moult. Removal of this hormone induces metamorphosis to the adult. It is not intended to discuss the role of these hormones in any detail here. In brief, the hormone analogues act as exogenous mimics of JH. As well, they may interfere with the biochemical mechanisms involved in regulation of secretion, transportation from the secretory site to target site, degradation, excretion and feedback control of the native hormones. Likewise, the negating of JH action using an anti-hormone of JH at a specific stage in the larval moult cycle forces a much earlier moult forming a sterile miniature adult pupa. Unlike the hormone analogues, overcoming the effects of the native hormones means that the destructive capacity of the larval instar stages is restricted. These analogues and anti-hormones are reviewed in more detail by Retnakaran *et al.* (1985) along with the benzoylphenylurea insecticides but it is obvious that the benzoylphenylureas are more flexible as IGR's as they do not rely primarily on hormonally-timed events to elicit an effect.

*Benzoylphenylureas*

The first IGR with activity to disrupt cuticle formation was synthesized at Philips-Duphar (van Daalen *et al.*, 1972) whilst attempting to produce a new herbicide. This was DU-19111, a combination of the herbicides diuron and dichlobenil from which the commercial compound Diflubenzuron (Dfb) was developed. This was the first of the group of compounds called the Benzoylphenylureas (BPU) as a product of renewed interest in chitin metabolism (Verloop & Ferrell, 1977). These interfere with the deposition of chitin (Mulder & Gijswijt, 1973) by affecting its synthesis (Post *et al.*, 1974). Some of the active compounds are outlined in Fig. 1.10. Quantitative Structure Activity Relationships (QSAR) have shown that the position of $Cl^-$ and $F^-$ atoms in the overall compound structure can greatly influence the toxicity of the IGR (Nakagawa *et al.*, 1984; Luteijn & Tipker, 1986; Nakagawa *et al.*, 1987) and that this toxicity is dependent on the substitution pattern in the benzoyl ring in the following order:

$$2,6\text{-}F_2 > 2\text{-}Cl > 2,6\text{-}Cl_2.$$

*Modes of action of the benzoylphenylureas*

Much effort has been put into investigating the precise biochemical mode of action of the benzoylphenylureas. There is no doubt that these compounds prevent the deposition of chitin in the cuticle. The protein content of *Schistocerca gregaria* cuticle does not change after Dfb treatment but the amount of chitin, measured as glucosamine after hydrolysis, is greatly reduced (Ker, 1977). On administration of the CS inhibitors UDP-GlcNAc has been found to accumulate (Hajjar & Casida, 1978: van Eck, 1979; Gijswijt *et al.*, 1979; Turnbull & Howells, 1982) suggesting interference with the polycondensation step leading to chitin. Using an *in vitro* system Post *et al.* (1974) found there was an accumulation of glucosamine using DU-19111 and UDP-GlcNAc using Dfb interpreting the result as enzyme-substrate inhibition with the latter completely

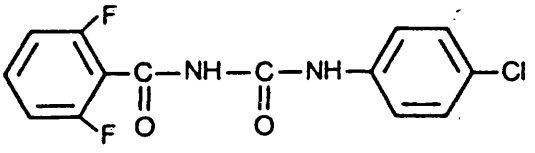
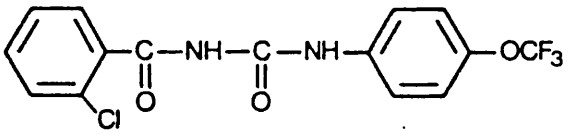
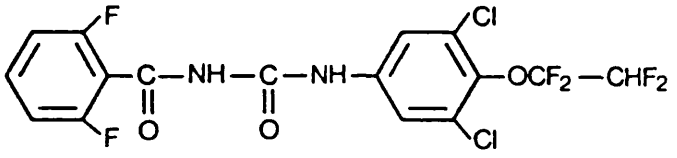
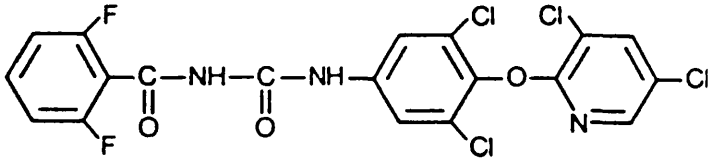
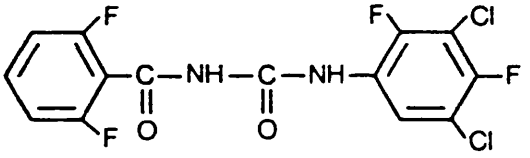
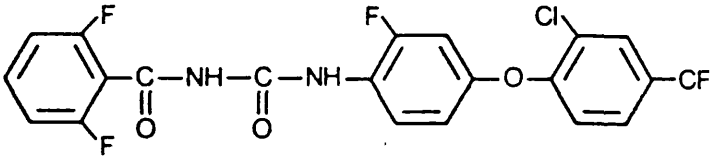
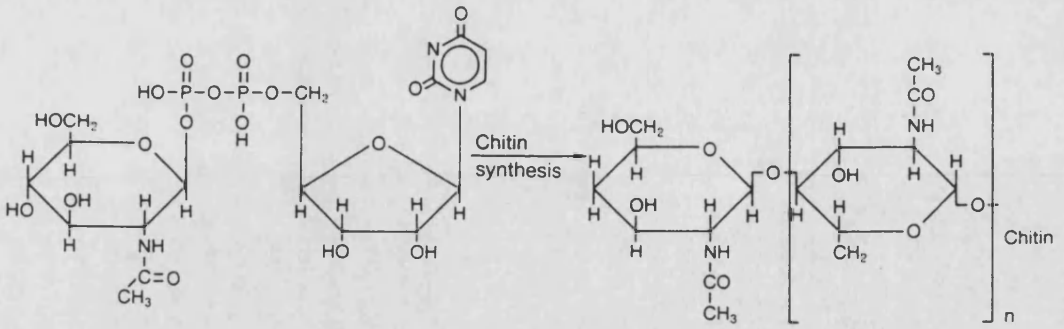
| Technical Name | Trade Name | Chemical Structure |
|---|---|---|
| **Diflubenzuron** | Dimilin |  |
| **Triflumuron** | Alsystin |  |
| **Hexafluoron** | XRD-473 |  |
| **Chlorfluazuron** | Atabron |  |
| **Teflubenzuron** | Nomolt |  |
| **Flufenoxuron** | Cascade |  |

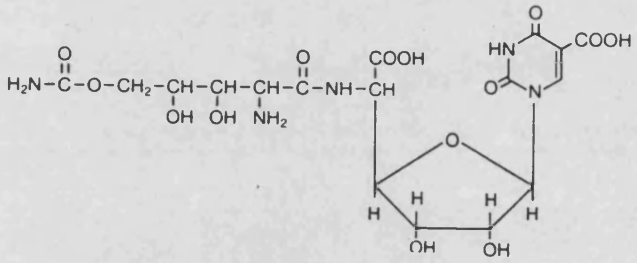
**Fig. 1.10.** Some Structures of Benzoylphenylurea Insecticides

blocking the enzyme and DU-19111 allowing binding of UDP-GlcNAc but not release of the hydrolysis product GlcNAc. This was at first thought to be due to direct inhibition of the CS complex (Post *et al.*, 1974) but insect cell-free systems showed this was not the case. Indeed van Eck (1979) showed caution saying "...final proof that chitin synthetase in insects is the target for benzoylphenyl ureas can only be given when chitin synthetase in insects can be isolated from insect tissue and studied *in vitro*." It has since been demonstrated that Dfb does not inhibit formation of chitin by insect tissues *in vitro* with UDP-GlcNAc as precursor (Cohen & Casida, 1980b; Mayer *et al.*, 1981), although antibiotics such as Polyoxin D and Nikkomycin Z do inhibit isolated CS in both fungi and insects. Both these compounds are structural analogues of the precursor UDP-GlcNAc (Fig. 1.11.). These analogues are thought to be competitive inhibitors of the CS enzyme (Gooday, 1972). In *Artemia salina*, the brine shrimp, Horst (1981) found Dfb inhibition of cell-free CS of 72-96% but Polyoxin D showed no inhibitory characteristics. Turnbull and Howells (1983) obtained some CS sensitivity with both Polyoxin D and Dfb. Both of these experiments are open to criticism. In the first case experimental detail was lacking casting doubt on it's validity as well as the nature of the enzyme studied. It may be that the crustacean CS is different to insect and fungal CS. In the latter, Dfb inhibition was only 50%, possibly being non-chitinous products (Reynolds, 1987).

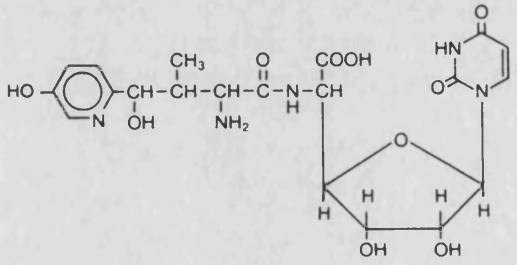
Although chitin synthesis inhibition in insects is the primary target using these insecticides it is clear from the known data that they do not act on the polymerization step. With acylurea inhibition of CS on the whole disproved other mechanisms of action have been proposed.




UDP-GlcNAc



Polyoxin D



Nikkomycin Z

**Fig. 1.11.** Diagram to show structural similarity between UDP-GlcNAc, Polyoxin D and Nikkomycin Z.

## Insecticidal Action Hypotheses

### *Modified Chitin Digestion*

Initially the lack of new cuticle after exposure to BPU's was ascribed to raised levels of chitinase (Ishaaya & Casida, 1974), the enzyme responsible for chitin degradation. Deul *et al.* (1978) found no increase in chitinase activity in larvae of *Pieris brassicae* and furthermore, chitin deposition was inhibited after as little as 15 minutes (confirming findings by Post *et al.*, 1974), too short a time span for raising levels of chitinase. The ecdysteroids stimulate chitinase from which Yu and Terriere (1977) concluded that Dfb inhibited the enzymes responsible for ecdysone degradation, suggesting Dfb was influencing the endocrine system (Lung, 1980). Against this, no difference was observed in the steroid titres of test (Dfb) and control pharate stable-flies at a stage known to have active β-ecdysone but the cuticle was still affected (O'Neill *et al.*, 1977).

### *Action on CS proenzyme*

Studies on fungi (Marks *et al.*, 1982) led to the theory that insect CS also exists as a proenzyme or zymogen activated by a chymotrypsin-like protease and that Dfb could inhibit the protease necessary for cleavage (Leighton *et al.*, 1981). However, it has not been shown that insect CS exists as a proenzyme although Mayer *et al.* (1980) showed trypsin to increase the original enzyme activity slightly.

### *Action on DNA synthesis*

DNA synthesis is reduced when cells are treated with Dfb (DeLoach *et al.*, 1981). Autoradiography showed no tritiated thymidine incorporation in to epidermal and tracheal cells after Dfb treatment which suggested that the properties of the membranes were modified thus changing the permeability to certain substances. Both cell types are capable of producing chitin. Meola and

Mayer (1980) found inhibition of thymidine incorporation into pupae of *Stomoxys calcitrans* (L.). Further evidence comes from observations of nucleoside uptake inhibition into cells from *Manduca sexta* (L.) (Klitschka *et al.*, 1986) and Harding-Passey melanoma cells (Mayer *et al.*, 1984). Whether these effects on DNA synthesis are primary or secondary remains to be resolved.

*Action on UDP-GlcNAc Transport*

Disrupted precursor access to the chitin synthase is another proposal for acylurea action but is only put forward by one group (Mitsui *et al.*, 1984, 1985). Using a gut sac preparation from *Mamestra brassicae* (L.), the incorporation of labelled UDP-GlcNAc into chitin was measured in the presence/absence of Dfb. Dfb inhibited incorporation when the labelled precursor was on the basal side of the gut epithelium but not when the labelled precursor was on the apical side of the epithelium (i.e. in the gut lumen). The location of the Dfb was unimportant. Mitsui *et al.* (1985) interpreted this experiment as follows: When the labelled UDP-GlcNAc is on the apical surface of the cell, it has direct access to the CS enzyme complex on the epithelial cell's apical membrane. When the labelled precursor is on the basal side of the epithelium, however, it must first be transported through the epithelial cells to reach this location. Dfb was supposed to prevent incorporation of the UDP-GlcNAc by inhibiting its transport across the cell membrane. As possible active transport mechanisms of UDP-GlcNAc the enzymes $Na^+$-$K^+$ and $Ca^{2+}$-$Mg^{2+}$ ATPases were assayed with Dfb but found to be insensitive. The transport inhibition interpretation has not yet found favour or been confirmed. A serious flaw in the argument is that UDP-GlcNAc is not in any case normally transported across the epithelial cells, but is formed within the epithelial cells. Since it is known that acylurea action causes a build-up of UDP-GlcNAc within chitin synthesising cells, the apparent inhibition of UDP-GlcNAc transport could be secondary to this accumulation.

*Inhibition of lipid-linked oligosaccharide intermediates*

Chitin synthesis is organised under enzymatic control and direct polymerization onto the growing crystallite (see Fig. 1.6). If chitin synthase is unaffected by acylureas then it may suggest that the BPU's inhibit that transfer of GlcNAc to the lipid carrier dolichyl phosphate in a similar manner to tunicamycin (Mayer *et al.*, 1981; Quesada-Allue, 1982). Tunicamycin is a nucleoside antibiotic which specifically blocks this biosynthetic process having no effect on chitin synthase (Cohen and Casida, 1980b; Mayer *et al.*, 1980). *in vitro*. Although the existence of these intermediates in insects has been demonstrated their role in chitin biosynthesis has not yet been defined (Hajjar, 1985).

*Active Acylurea Metabolites*

Knowing that insect CS is insensitive *in vitro* to Bay Sir 8514 led Cohen and Casida (1980b) to hypothesise that it was potent metabolites which inhibit the enzyme. However, attempts to extract such metabolites from *Tribolium castaneum* previously given the insecticide no such inhibition of CS (Cohen & Casida, unpublished; cited in Cohen and Casida, 1980).

*End Chain Disruption*

A possible explanation of acylurea action comes from Reynolds (1989) although proof of such action would be hard to obtain. He supposes that the chitin synthase complex is not, as normally supposed, composed of only a single enzyme. He argues that because alpha-chitin has equal numbers of anti-parallel chains, for every addition of a 1'-activated GlcNAc monomer (UDP-GlcNAc) to the microfibre in the β 1-4 orientation there must also be an equal addition of a novel 4'-activated monomer to a microfibre in the β 4-1 orientation. Measuring the incorporation of UDP-GlcNAc might not reveal disruption of the second process. This hypothesis remains untested.

**Morphology of Disruption**

Since the incorporation of chitin into cuticle is impaired on exposure to acylurea compounds it follows that the appearance of the cuticle will also differ from normal insects. Soon after treatment the most noticeable change is in the appearance of the endocuticle (Binnington, 1985; Hassan & Charnley, 1987). Electron-dense deposits (thought to be proteinaceous) arise from the epidermal cells. The apical microvilli from which they seem to arise regain their normal appearance after extruding the material. Cuticle laid down after insecticide treatment loses its regular lamellate order so that, post-treatment, the number of lamellae does not increase (Hassan and Charnley, 1987). Indeed, using a sublethal dose, chitin synthesis can eventually re-establish itself producing a disrupted band within an otherwise normal cuticle (Ker, 1978). Using Wheat Germ Agglutinin (WGA) as a cytochemical marker for GlcNAc it soon becomes apparent that there is none of this amino sugar present in the treated cuticle (Lee *et al.*, 1990). This last finding simply supports the idea that acylureas really do inhibit chitin synthesis, and do not merely cause the synthesis of poorly ordered chitin microfibrils.

**The Aim of This Project**

The mechanism by which acylurea insecticides act has still to be elucidated. Different results from various groups give no real indication of how these compounds interact within the insect cell and whether the observed effects are primary or secondary.

This project has attempted to define more clearly the morphological and biochemical changes seen after exposure to these insecticides and, moreover, to make initial steps towards identifying a potential acylurea receptor within the epidermal cell. It seems likely that the true subunit structure of the insect chitin

synthase and acylurea receptor will be elucidiated by the techniques of molecular biology, although it will be essential to back such work with biochemical evidence. Characterization of acylurea-induced morphological and biochemical changes are a step in this direction.

# Chapter 2

## Effects of Flufenoxuron on the tobacco hornworm, *Manduca sexta*, *in vivo* and *in vitro*

### Introduction

Flufenoxuron is a member of the acylurea (benzoylphenyl urea) class of insecticides introduced by Shell as "Cascade" in 1989. As discussed in Chp. 1., diflubenzuron, the prototype of this class is now generally accepted to cause death through the inhibition of chitin synthesis (Post *et al.*, 1974). Other members of the class have been shown to also inhibit chitin deposition, including flufenoxuron (Clarke and Jewess, 1990a).

This chapter describes in some detail the effects of flufenoxuron on a model Lepidopteran insect, the tobacco hornworm *Manduca sexta*. Also described is the development of an *in vitro* assay for chitin synthesis by explanted integumental tissue of Manduca, and the effect of fluenoxuron on it.

### Materials and Methods

Tobacco hornworms, *Manduca sexta* (L) (Lepidoptera: Sphingidae), were reared on artificial diet (see Appendix A) according to standard methods (Bell and Joachim, 1976). The main culture was kept at 25$^{o}$C under a long-day photoperiod regime (LD, 17:7). Day 0 5th larvae (12-13 days post-hatch) were used in all the experiments except where indicated.

*Chemicals*

The insecticide was technical grade. Flufenoxuron (WL 115110) was a gift from Shell Research Ltd, Sittingbourne, Kent, UK. [U-$^{14}$C]-Glucose (230mCi mmol$^{-1}$) and N-acetyl-D-[1-$^{14}$C]-glucosamine (GlcNAc, 58.7 mCi mmol$^{-1}$) were from Amersham International, Amersham, UK. Grace's medium was obtained from GIBCO, Paisley, Scotland. All other reagent were of analytical grade from either Sigma (Poole, Dorset, UK.) or BDH (Poole, Dorset, UK.).

*Effect of flufenoxuron on the rate of development and mortality of fourth stadium Manduca sexta*

Samples each consisting of ten freshly ecdysed fourth stadium *Manduca* larvae were subjected to a range of concentrations of flufenoxuron. These doses were administered by either incorporating flufenoxuron (dissolved in acetone) into the artificial diet or by injection into the larva. 100 ml of artificial diet was required to treat a sample of 10 larvae. After the diet had cooled to 70$^{o}$C, but before it had set, 0.1 ml of the acetone/flufenoxuron solution of a predefined concentration was mixed in so as to give the required final concentration of flufenoxuron (see below). 10 ml of treated diet replaced the existing diet and would last the larvae throughout the fourth stadium. Controls were given diet containing 0.1% acetone.

For injection, a solution of flufenoxuron in acetone was rapidly mixed with a simple insect saline ('E&B' saline: Ephrussi and Beadle, 1936)(see Appendix A) to form a suspension. The suspensions were no more than 5% acetone. Prior to the injection the larvae were anaesthetised by placing in water for 20 min. The injection was through a posterior intersegmental membrane using a Hamilton syringe and an SWG needle and, post-injection, the insects were surface sterilised by briefly dipping in 70% ethanol. The suspensions were made up freshly for each session of injections to ensure that no precipitation of flufenoxuron occurred. The concentrations tested were 0.01 to 0.10 ppm (w/v) of

flufenoxuron. The larvae were removed from their containers daily and weighed. This was continued until the larvae wandered in the fifth stadium. Mortality was also recorded on a daily basis.

*Effect of flufenoxuron on Uptake of [$^{14}$C]-Glucose in vivo*

Day 0 5th larvae were injected with 10 µl of E&B saline containing 5% acetone (controls) using a Hamilton syringe and a 28 SWG needle using the above procedure. For treated larvae, stock flufenoxuron in acetone was rapidly mixed with E&B saline giving a final concentration of 10 µg $10^{-1}$ µl. These injections were given 3 h prior to an injection of [$^{14}$C]-Glucose (0.1 µCi in 10 µl E&B) and larvae were then returned to the holding temperature of 25°C. After 1 h at 25°C the larvae were frozen on dry ice, tissue between the 4th and 7th segments removed, and the gut taken out. This abdominal sample was washed in distilled water ($dH_2O$) and again in 100% ethanol, finally being carefully wiped to remove excess fat and muscle. Cuticles were deproteinised overnight with 3 ml 1 M KOH at 80°C, and once cool, washed several times in 250 ml $dH_2O$. These were then dried in an oven (60°C) for 24 h.

The KOH-insoluble product, once dried, was digested by 0.05 mg *Streptomyces griseus* chitinase (10-15 U $mg^{-1}$, Sigma) in 0.5 ml citrate/phosphate buffer (0.2 M, pH 6.0) overnight at 25°C in a sealed scintillation vial. Radioactivity was counted by adding 9.5 ml scintillation cocktail (LKB Optiphase 'Safe') to the chitinase digest and counted with an LKB 1217 Rackbeta counter.

*In vitro uptake of [$^{14}$C] Radiolabelled precursors into M. sexta Epidermis and Cuticle*

Many initial experiments were carried out to establish a useful *in vitro* technique for measuring chitin synthesis. One question was: Which was the best assay tissue? Prolegs were found to be a suitable quantitative tool. They were

removed from the body by cutting along the proleg/body infold. For size consistency only the rear prolegs were used. Excised prolegs were placed in a petri dish containing fresh *Manduca* saline (see Appendix A) until required.

Initially, to compare the compatibility of the insect media, prolegs were incubated in 100µl *Manduca* saline (containing a few crystals of phenylthiourea, PTU, to inhibit tyrosinase activity and so prevent tissue darkening) or Grace's medium (see Appendix A) in the presence of 0.1µCi [$^{14}$C]-GlcNAc (1.7 nmol). Prolegs were maintained at 25°C in Eppendorf tubes for 1 or 3 h and the incorporation stopped by briefly washing in the appropriate cold medium and then placed in Sarstedt tubes containing 0.3 ml 50% KOH at 100°C (van Eck, 1979). This temperature was maintained for 30 min using a Dri Block (Techne OB3). KOH-insoluble material was counted for radioactivity without further processing after extensive washing in $dH_2O$ by placing the material in a scintillation vial containing 5 ml scintillation fluid and counted as previously mentioned. KOH-soluble material plus 0.7 ml $dH_2O$ from the washed tube was also monitored for [$^{14}$C] radioactivity by the addition of 9 ml scintillation fluid.

The effect of aerating the incubation medium was assessed by bubbling medical $O_2$ through *Manduca* saline via an aquarium stone. After an hour of aeration the above assay procedure was repeated again for 1 or 2 h in the presence of [$^{14}$C]-GlcNAc. Radioactivity was measured as previously outlined.

Having optimized the assay environment, [$^{14}$C] precursor preference was studied by comparing uptake of either [$^{14}$C]-Glucose or [$^{14}$C]-GlcNAc (0.1µCi in 100 µl of medium) into KOH-soluble and insoluble fractions. Excised rear prolegs were processed after 1 or 2 h.

*Time Course of [$^{14}$C]-GlcNAc Incorporation*

Rear prolegs from anaesthetised day 0 5th larvae were removed and placed into Eppendorf tubes containing 0.1μCi GlcNAc in 100μl *Manduca* saline previously aerated for 1 h. Tubes were maintained at 25°C in a water bath for increasing times (5 min - 3 h). After KOH digestion, the KOH-insoluble (chitin) and KOH-soluble ( protein ) fractions were measured for radioactivity.

*Uptake of [$^{14}$C]-GlcNAc into pharate 5th larvae*

Rear prolegs were removed at the stages of either tanned or non-tanned mandibles (as seen through the slipped headcap) pharate 5th instar larvae. The prolegs were incubated in *Manduca* saline (0.1μCi [$^{14}$C]-GlcNAc 100$^{-1}$μl) either with or without the old, overlying 4th stadium cuticle attached. Tissue was incubated for 2 h (25°C) before being processed as previously outlined.

*In Vitro Dose-Response for Flufenoxuron Inhibition of [$^{14}$C]-GlcNAc accumulation in 5th stadium larvae*

Stock solutions of flufenoxuron were made using dimethyl sulphoxide (DMSO) as the solvent system and were protected from light to prevent possible photodegradation. Rear prolegs from Gate 2 (Truman, 1972) larvae were first preincubated (15 min) in 100μl *Manduca* saline containing either (control) 0.5% DMSO, or (experimental) flufenoxuron (0.082 - 10.2 μM) in 0.5% DMSO. Subsequently the prolegs were transferred to identical solutions containing 17 μM [$^{14}$C]-GlcNAc (0.1 μCi) for 1 h. Incubations were terminated as described.

*Enzymatic test for Chitin*

To test for chitin, prolegs from day 0 5th larvae were labelled with [$^{14}$C]-GlcNAc (0.1μCi in 100 μl, 2 h) and treated as described above. Cuticular chitin was measured by a method adapted from Gelman and Borkovec (1986). The KOH hydrolysate (both soluble and insoluble) was neutralised with 5N HCl and filtered

on a Millipore tower to trap the particulate matter on GF/B (Whatman) filters. These were washed with 10 ml $dH_2O$ and then half of the filter papers with radioactive chitin were transferred to scintillation vials containing citrate/phosphate buffer (0.2 M, pH 6.0) and 800µl chitinase (2.5 mg $ml^{-1}$). These were incubated for 60 h at 25°C. The rest of the filter papers (controls) were incubated in a similar manner in buffer without chitinase. After incubation, all the samples were refiltered and the vials were washed successively with 10 ml $dH_2O$, 10 ml 95% ethanol and 10ml $dH_2O$. For each sample the new and original filters, once dried, were counted together in 5 ml scintillation fluid.

## Results

*Insecticidal effect of Flufenoxuron*

Flufenoxuron is lethal to *Manduca* larvae when given to the insects during the fourth stadium in their food. Counting all mortality up to the time of wandering, the $ED_{50}$ for flufenoxuron was 0.045 ppm (w/v) (Fig. 2.1.).

Some insects died at the time of the 4th to 5th stage moult, but a prominent component of the effect of flufenoxuron was the inhibition of growth during the fifth stadium (Fig. 2.2.). Growth rate following ecdysis to the fifth stadium was in fact inversely proportional to the concentration of flufenoxuron in the diet (Fig. 2.3.), despite the fact that treatment of the diet with insecticide was discontinued at the end of the fourth stadium. Insects given flufenoxuron-treated diet continued to die during the fifth stadium. In general, the higher the concentration of insecticide in the diet, the earlier that death occurred, so that there was an inverse relationship between the weight of the insects one day before they died and the concentration of flufenoxuron (Fig. 2.4.).

Injections of flufenoxuron also caused mortality. When varying doses of the insecticide were given on day 3 of the fourth stadium,the $LD_{50}$ was 0.11 $\mu g\ g^{-1}$ (Fig. 2.5.). The differing sensitivity of the larvae to flufenoxuron at different stages of the moulting cycle was assessed by giving a single injection of flufenoxuron on a particular day of the fourth stadium (Table 2.1.). The insects were least sensitive to the insecticide on day 1 of the fourth stadium, and were most sensitive on day 0, just after ecdysis to the fourth stage, and again on day 3, just before initiating the moult to the fifth stadium. Interestingly, the immediate cause of death varied with the age of treatment. Insects given a single injection of flufenoxuron on days 0, 1 and 2 of the fourth stadium were most likely to die as a result of their failure to grow during the fifth stadium. Those insects given the insecticide on days 3 or 4 of the fourth stadium, on the other hand, were more likely to rupture the new cuticle during ecdysis from the fourth to fifth stage. On any one day of the fourth stadium, a larger dose of insecticide was more likely to cause death by cuticular rupture than was a smaller dose.

*Inhibition of Chitin Synthesis by flufenoxuron*

*Chitin Synthesis in vivo*

Fifth stadium larvae were used for this, in order to give sufficiently large amounts of integumental tissue. Day 0 larvae were used because Mitsui *et al.* (1980) had previously shown that chitin synthesis was maximal at this time.

An initial experiment demonstrated that, as expected, flufenoxuron inhibited the incorporation of a labelled precursor into cuticular chitin (Table 2.2.). A single injection of 100 ng flufenoxuron into day 0 fifth stage *Manduca* larvae (a dose of approximately 0.065 $\mu g\ g^{-1}$) caused 76% inhibition of [$^{14}$C]-Glucose incorporation over a 1 h period. Further work necessitated the development of an *in vitro* system, however.

*Chitin Synthesis in vitro*

Prolegs were selected for further study of *in vitro* chitin synthesis because this structure contains very little non-integumental tissue. It was necessary to investigate the effects of experimental conditions on rates of incorporation of precursors in order to optimise the assay.

First, the precursors [$^{14}$C]-Glucose and [$^{14}$C]-GlcNAc were compared (Table 2.3.). Incorporation of [$^{14}$C]-GlcNAc occurred far more efficiently, and so was used in all further experiments.

Second, the effect of incubation medium was investigated. A comparison of *Manduca* saline solution with Grace's insect tissue culture medium showed that incorporation, over a 2 h test period, was more rapid in the *Manduca* saline solution (Table 2.4.). Therefore, further experiments used the simpler *Manduca* saline.

Third, the requirements of *in vitro* chitin synthesis for oxygen was assessed. It was discovered that active aeration of the assay medium immediately prior to the assay significantly improved incorporation of the labelled precursor into chitin (Table 2.5.). Further experiments therefore employed oxygenated media.

The time course of incorporation of [$^{14}$C]-GlcNAc was investigated (Fig. 2.6.). Incorporation into chitin was found to be linear from 1 - 3 h, but was subject to an initial time lag of up to 1 h during which the rate of incorporation increased with time. Incorporation into the KOH-soluble, non-chitin fraction increased rapidly during an initial 30 min period, but increased much more slowly after this time. Presumably the initial delay in the incorporation of [$^{14}$C]-GlcNAc into

chitin represents the period necessary to achieve uniform labelling of the pool of chitin precursors within the explanted tissue.

The identity of the labelled KOH-insoluble fraction as chitin was confirmed by enzymatic digestion. 88% of the KOH-insoluble radioactivity was released by treatment with chitinase as compared to controls. The results verified that the radioactivity counted was due to radiolabelled chitin and not due to non-specific binding to the filter discs.

The effect of flufenoxuron on chitin synthesis *in vitro* by explanted prolegs could now be investigated. The insecticide was found to inhibit chitin synthesis over a range of concentration from 0.04 - 25 $\mu g\ ml^{-1}$ (0.082 - 10.2 $\mu M$). The $IC_{50}$ (concentration required to inhibit synthesis by 50%) was approximately 0.2 $\mu g\ ml^{-1}$, or 0.4 $\mu M$ (Fig. 2.7.).

*Cuticular Permeability to Precursor*

The incorporation of [$^{14}$C]-GlcNAc into pharate 5th instar larval cuticle using explanted proleg material was investigated. This was done in order to assess the availability of [$^{14}$C]-GlcNAc in the *Manduca* saline solution for incorporation into the new cuticle of the 5th stadium larva. Having removed the proleg tissue the experiment proceeded in two ways using larvae with either tanned or non-tanned mandibles.

First, the removed pharate prolegs were incubated with the 4th stadium cuticle still attached. It was found that precursor incorporation into KOH-insoluble material taken from pharate 5th larvae bearing non-tanned mandibles was, on the average of two experiments, more than double the amount of radioactive counts (Table 2.6.) found in larvae bearing tanned mandibles.

Second, the pharate prolegs were incubated with the overlying 4th cuticle removed. It was found that the accumulation of [$^{14}$C]-GlcNAc was far greater in both the chitinous and KOH-soluble fractions than when the old 4th stadium cuticle was left intact. This was seen for both the tanned and non-tanned states (Table 2.6.).

**Discussion**

Flufenoxuron is an excellent insecticide against fourth stadium *Manduca* larvae. Fourth stadium caterpillars given flufenoxuron in their food were killed by the insecticide either at the time of ecdysis to the fifth stadium or during the subsequent stage. Where the insects were killed at the moult this was because they were unable to shed the exuvia, frequently rupturing the new cuticle in their efforts to escape, and suffering lethal loss of body fluids. Even when the old cuticle was shed, the affected insects often retained a 'ligature' of old cuticle around the terminal abdominal segments which constricted later growth. Those affected insects successfully escaping the old fourth stage cuticle frequently failed to thrive in the next stage even though the insecticide was now withdrawn. Slowed rate of growth was strongly correlated with the dose of insecticide received in the previous stadium. Death occurred either as a result of rupture of the body wall during the fifth stage, in the case of those insects that displayed significant growth in size, or without such external symptoms in the case of those insects that did not grow, presumably as a result of starvation. The size of the insects at the time of death was strongly inversely correlated with the dose of insecticide received.

These symptoms of poisoning are consistent with the presumed mode of action of flufenoxuron, as a member of the acylurea class of insecticides, of inhibiting the deposition of cuticle chitin (see Chp. 1.). The chitinless cuticle so

formed in the affected insects is too weak to survive ecdysis, or if the moult is successfully achieved, is too weak to allow further growth in the next stage. Growth during the treated stage is hardly affected because the already existing cuticle is sufficiently strong to support growth during that stadium.

The failure of treated insects to thrive in the next stage is interesting. A number of authors have commented on this so called 'anorexic' effect of acylureas (Retnakaran *et al.*, 1985; Neumann and Guyer, 1987). This effect is probably due to the malformation of the next stadium's mouthparts. The affected, 'anorexic' insects attempt to feed, but are unsuccessful in acquiring food (P.J. Jewess, *pers comm*).

It is clear from data presented here that 'failure to thrive' in the stage following treatment is a major cause of flufenoxuron-caused mortality in *Manduca*, as it probably is in other insects too. For example, when flufenoxuron was injected into day 3 fourth stage insects at the $LD_{50}$ of 0.11 µg, more than half of the mortality observed was of this type. When the insecticide was given earlier in the fourth stadium, on day 0, all of the mortality observed was due to 'failure to thrive'. Inspection of the data of Table 2.1. shows that this type of mortality is more common when the dose of insecticide is low. Higher doses lead to a higher death rate at the time of ecdysis.

The differing sensitivity of *Manduca* larvae to flufenoxuron on different days of the fourth stage is probably best explained in terms of the formation of the fifth instar cuticle, which does not occur until the fifth day of the fourth stadium. Mortality during ecdysis is more likely to occur when flufenoxuron is given late in the fourth stage, at or close to the time when fifth instar cuticle chitin is being synthesised. When the insecticide is given early in the fourth stage, some time previous to the stage of active fifth instar cuticle deposition, this kind of mortality

is observed less frequently, as though a lower dose of insecticide had been given. Presumably what is happening here is that the injected flufenoxuron is lost from the body, either because it is excreted, metabolised or sequestered. Clarke and Jewess (1990b) have shown that in *Spodoptera littoralis* 6th stadium larvae, flufenoxuron is subject to a rather slow, but nevertheless significant, metabolism to amide and benzoic acid derivatives. Additionally, these authors found that when flufenoxuron was applied topically, of the insecticide that entered the body, approximately half was present in the gut. This implies that with time, injected insecticide (as used in the present experiments) would be eliminated via the faeces. We have not investigated this point directly in *Manduca*, however.

It was expected that flufenoxuron would prove to be a potent inhibitor of chitin synthesis. The symptoms of poisoning of *Manduca* larvae are clearly consistent with this as a mode of action, and are extremely similar to the symptoms of poisoning by other acylureas (see Chp. 1.). At the time at which this this work was done there was no published reports of chitin synthesis inhibition by flufenoxuron, although recently, papers by researchers at Shell Research Ltd (Clarke and Jewess, 1990a; 1990b; Lee *et al.*, 1990) have established that flufenoxuron inhibits chitin synthesis in larvae of the cotton leafworm, *Spodoptera littoralis*. They found that topically applied diflubenzuron, flufenoxuron or teflubenzuron were all inhibitors of [$^{14}$C]-GlcNAc incorporation during a 5 h test period.

An *in vivo* experiment employing [$^{14}$C]-Glucose as a radiolabelled precursor for chitin synthesis demonstrated, as expected, that flufenoxuron inhibits chitin synthesis. However, we wished to develop an *in vitro* assay for chitin synthesis that would allow a better control of assay conditions and of precursor pool sizes, so that we could investigate the biochemical mode of action of the insecticide (see later chapters).

Several experiments that were performed in order to optimise assay conditions are described in this chapter. It is of particular interest that better results were obtained using the relatively simple *Manduca* saline than the very complex Grace's tissue culture medium. Incorporation was linear up to 3 h implying that the simpler medium is quite adequate to support the epidermal cells in a suitable state for cuticle deposition during this time. The lower rate of incorporation found with Grace's may reflect that medium's content of metabolic intermediates which may feed into precursor pools and thus dilute the labelled precursor's contribution to those pools.

The requirement for active oxygenation to obtain the best rates of chitin synthesis has not been previously noted. It should not be surprising that insect cells, normally supplied by the tracheal system with $O_2$ at a high partial pressure, should function more effectively when given extra oxygen. In the only previous examination of this question that has been found, Vardanis (1976) found that keeping grasshopper integumental tissue under an $O_2$-enriched atmosphere did not enhance chitin synthesis.

The incorporation of [$^{14}$C]-GlcNAc into chitin was much more efficient than the incorporation of [$^{14}$C]-Glucose. Several authors have previously commented that this is the case (Fristrom, 1968; Gwinn and Stevenson, 1973a; Christiansen *et al.*, 1984). It is interesting to note the 'classical' pathway for chitin synthesis as introduced by Candy and Kilby (1962) and reproduced in many texts (e.g. Neville, 1975; Hackman, 1984) fails to mention GlcNAc as a precursor for chitin. Since there is general agreement (see Chp. 1.) that UDP-GlcNAc is the final intermediate in chitin biosynthesis, GlcNAc must be converted directly or indirectly to UDP-GlcNAc. Since it is incorporated with higher efficiency than glucose, it is reasonable to infer that this conversion is more direct than that of

glucose. Gwinn and Stevenson (1973b) showed that crayfish integument contained a GlcNAc kinase and a UDP-GlcNAc pyrophosphorylase that would effect this conversion. Presumably the epidermal cells of other Arthropods also contain these enzymes.

It is not clear whether the normal pathway for chitin synthesis would include GlcNAc. It does not follow simply that because labelled GlcNAc can be efficiently incorporated into chitin that this is the normal route. However, it is likely that GlcNAc is readily available to the epidermal cells at the time of moulting since enzymatic breakdown of chitin by moulting fluid enzymes produces GlcNAc as its end product. Gwinn and Stevenson (1973a) established that, during the synthesis of new crayfish cuticle within the pharate stage, over half of the cuticular material incorporated into new cuticle came from the previous stage cuticle which had received radiolabelled [$^{14}$C]-GlcNAc. On the other hand, an *in vitro* study by Surholt (1975) established that, within the larval-adult moult of *Locusta migratoria*, glucose and not GlcNAc was the preferred precursor although both precursors were incorporated. Essentially both studies showed that within the pharate larva, GlcNAc was utilised for new cuticle synthesis, demonstrating that, as mentioned above, GlcNAc may act as a chitin precursor by way of conservation of cuticular material through enzymatic digestion but only during the moult cycle. Strangely, although Gwinn and Stevenson (1973a) suggested GlcNAc was the major premoult precursor (also confirmed by Christiansen *et al.*, 1984), this present study has shown it (GlcNAc) to be the major precursor in postmoult synthesis of cuticle. In addition to the reutilisation of old chitin, substantial amounts of totally new chitin must be produced in the postmoult phase to allow for larval growth. Presumably the pathway for the *de novo* synthesis of this chitin would proceed from glucose (see Fig. 1.6., Chp. 2.). It may be that the lower efficiency of glucose incorporation into chitin is due to

the presence of competing synthetic pathways that utilise glucose, but not GlcNAc (glycogen synthesis might be one such pathway).

The study of pharate 5th instar larvae revealed higher incorporation of the precursor GlcNAc when the old cuticle was removed (Table 2.6.). The results presented suggest that the underlying cuticle produced at apolysis is very permeable to chitin precursors. This is of economic importance to the larva because it suggests that the old cuticle can be enzymatically recycled (Wigglesworth, 1984) to form new cuticle by the passage of its breakdown products through this permeable cuticle. This route may be additional to the alternative proposal that digested cuticular precursors are recycled by the pharate larval instar "drinking" the moulting fluid (Zacharuk, 1972). At all events, the epidermal cells synthesizing the new cuticle are exposed to precursor, from both apical and basal sides, presumably maximising the supply.

Pharate cuticle at the tanned mandible stage with the overlying 4th stadium cuticle removed incorporated less GlcNAc than identically treated untanned cuticle. When one again regards the process of reabsorption through the cuticle, the epicuticle will only allow small molecules through it (according to Locke, 1966, the cuticulin layer has pores only 3 nm in diameter) by restricting access through the pore canals. Obviously, once tanning of the pre-ecdysial cuticle occurs, then transport of precursors would be reduced due to extra cross-linking within the chitin-protein matrix.

Many *in vitro* systems have been developed to test for chitin synthesis inhibition by the benzoylphenylurea insecticides, of which diflubenzuron was one of the first. These systems have included organ culture (Sowa and Marks, 1975; Vardanis, 1976) and cell-free systems (Cohen and Casida, 1980a; Marks *et al.*, 1980). Previous *in vitro* studies with diflubenzuron were found to inhibit chitin

synthesis with reported $IC_{50}$ values ranging from 7 x $10^{-7}$ M (Turnbull and Howells, 1982) to 1.1 x $10^{-9}$ M (Mitsui *et al.*, 1980). The basic *in vitro* assay described here produces inhibition at a similar molar concentration (4.1 x $10^{-7}$ M) and is potentially therefore a viable tool in the process of evaluating new insecticide formulations.

To conclude, it has been established in this chapter that flufenoxuron can inhibit incorporation of radiolabelled chitin precursor and hence, chitin synthesis in *Manduca* larvae. This is in agreement with the findings of researchers at Shell Research Ltd (Clarke and Jewess, 1990a; Lee *et al.*, 1990) working with *Spodoptera littoralis*. However, to derive information about the mode of action of flufenoxuron on the molecular level requires further work. This is outlined in subsequent chapters.

**Fig. 2.1.** The overall mortality response of 4th stadium *Manduca sexta* larvae, from day 0 onwards, to different doses of flufenoxuron in artificial diet (n=10 larvae) per point.

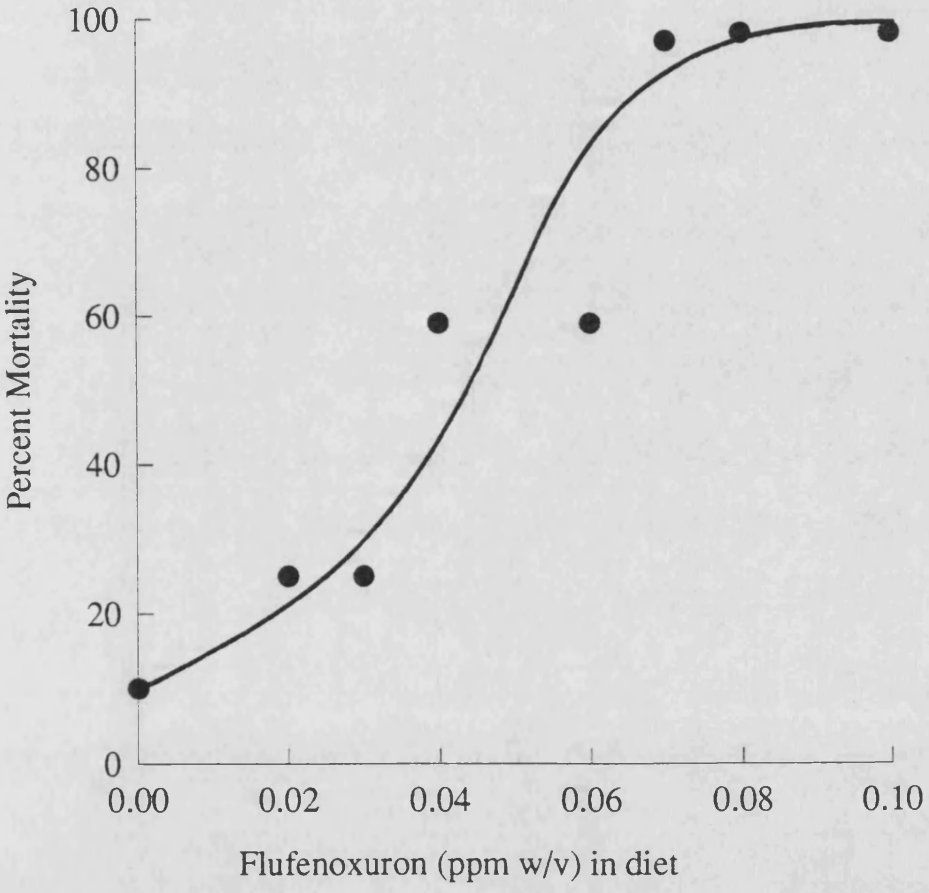

100
80
60
40
20
0
Percent Mortality
0.00
0.02
0.04
0.06
0.08
0.10
Flufenoxuron (ppm w/v) in diet

**Fig. 2.2** The effect of flufenoxuron (ppm w/v in diet) on growth rate of fourth stadium *Manduca sexta* larvae from day 0 through to the fifth stadium (moulting to fifth stadium occurs on day 4 since start of stadium). n=10 per point.

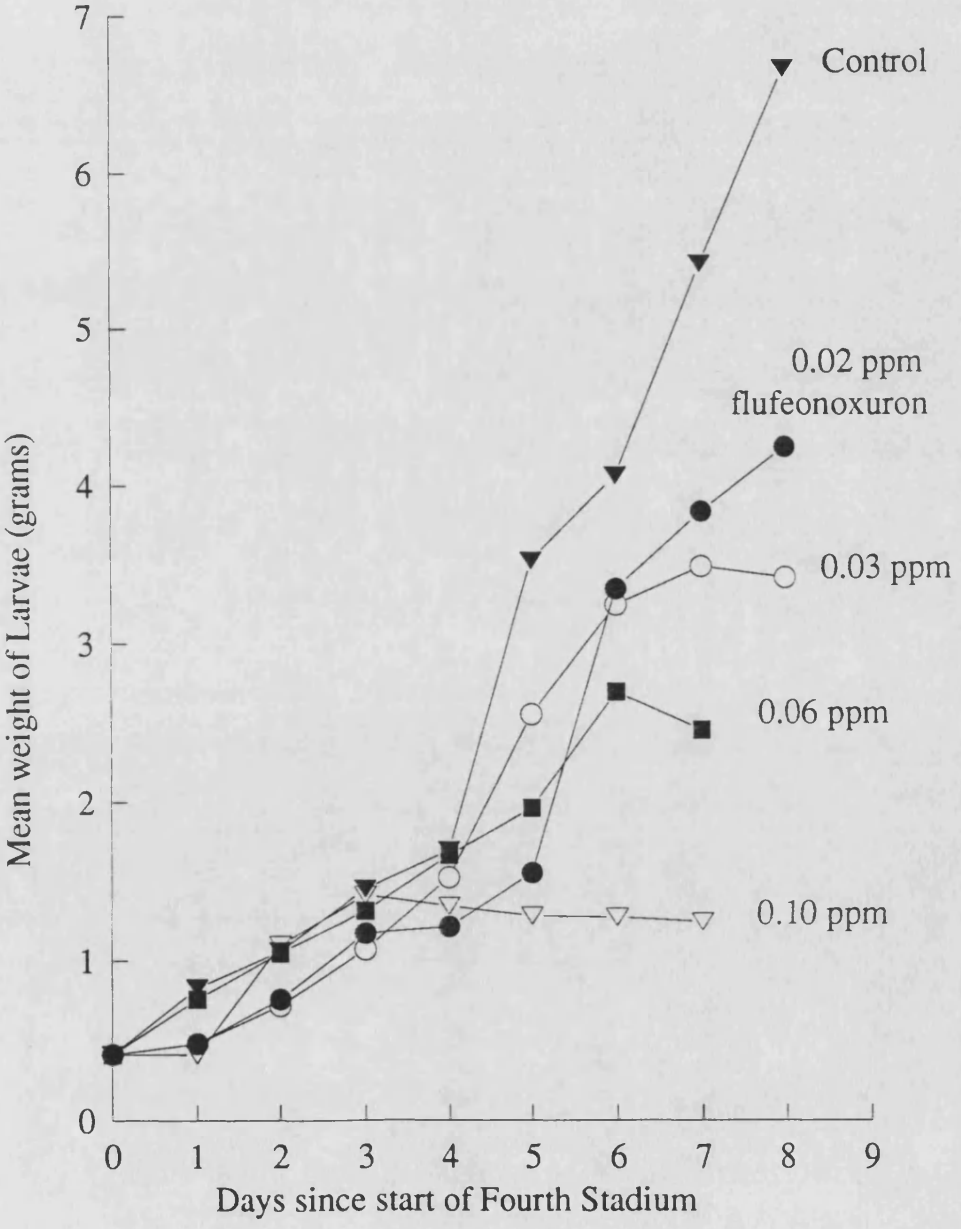

Mean weight of Larvae (grams)
0
1
2
3
4
5
6
7
0
1
2
3
4
5
6
7
8
9
Days since start of Fourth Stadium
Control
0.02 ppm
flufeonoxuron
0.03 ppm
0.06 ppm
0.10 ppm

**Fig. 2.3.** Rate of weight gain of surviving 5th stadium *Manduca sexta* following flufenoxuron treatments during the fourth stadium (as for Fig. 2.2.). n=10 per point. Regression analysis does not include point at 0.01 ppm.

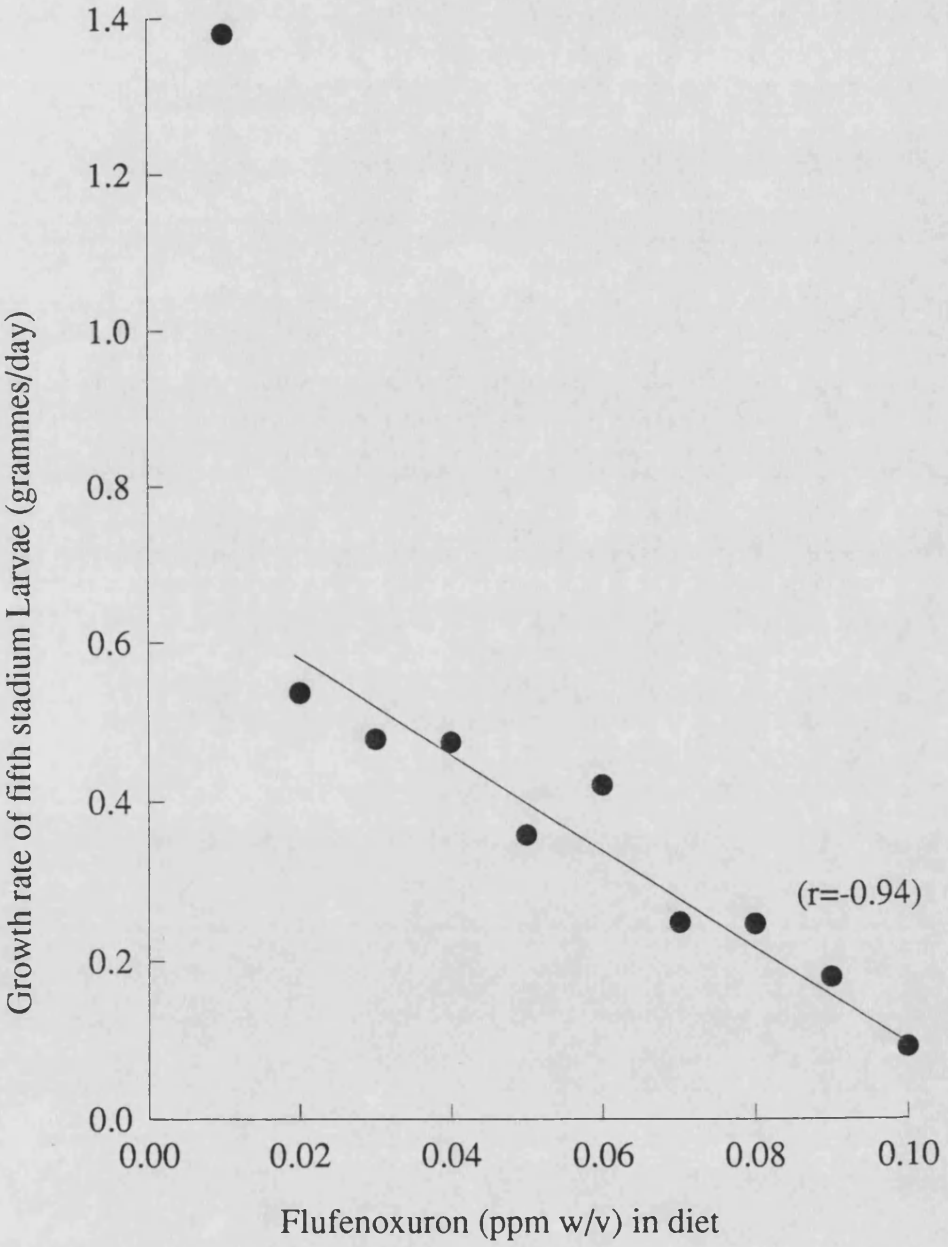

Growth rate of fifth stadium Larvae (grammes/day)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.00
0.02
0.04
0.06
0.08
0.10
(r=-0.94)
Flufenoxuron (ppm w/v) in diet

**Fig. 2.4.** Mean weight of fifth stadium *Manduca sexta* one day before death against flufenoxuron concentration (fourth stadium treatment - see Fig. 2.1.). n=10 per point. Means $\pm$ S.E.M. Line drawn by eye.

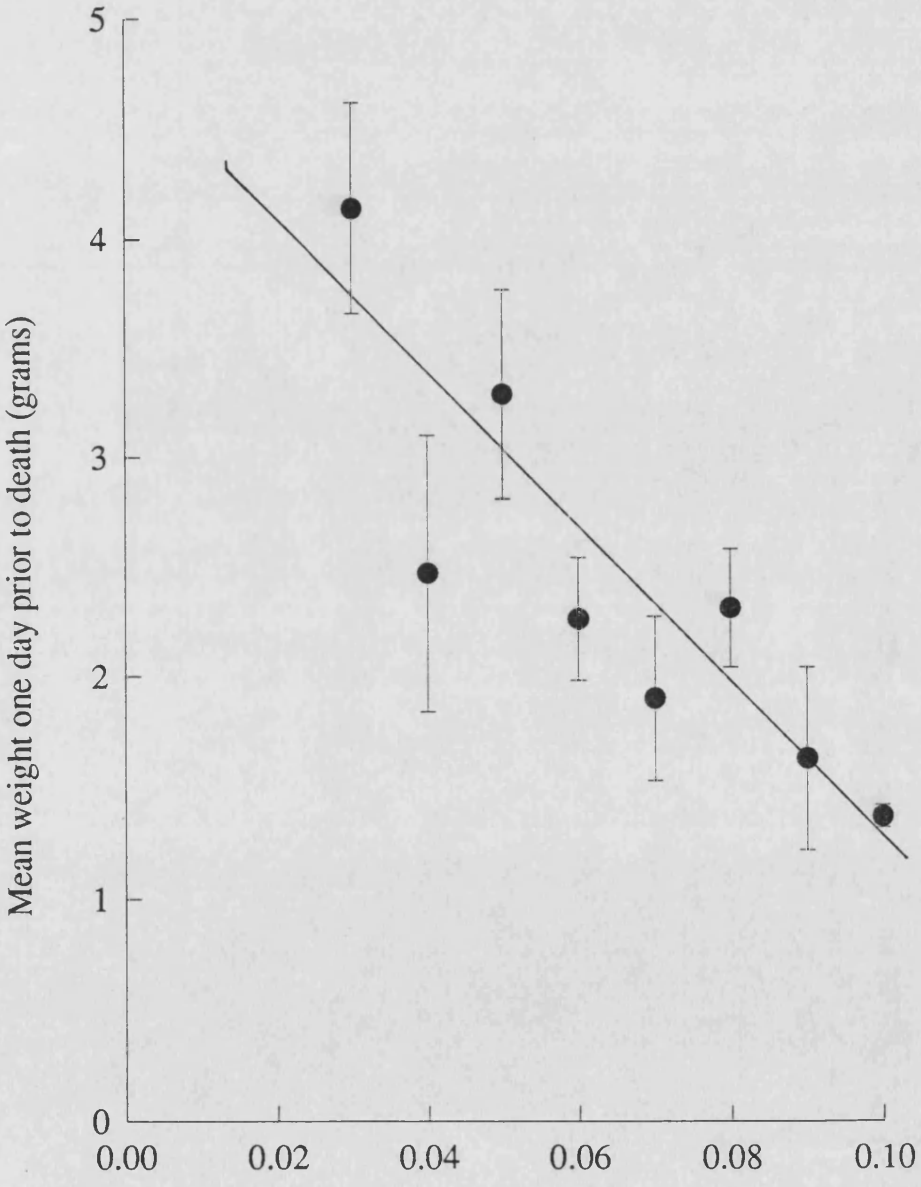

Mean weight one day prior to death (grams)
5
4
3
2
1
0
0.00
0.02
0.04
0.06
0.08
0.10
Flufenoxuron (ppm w/v) in 4th Stadium diet

**Fig. 2.5.** The mortality response of day 3 fourth stadium *Manduca sexta* to injected doses of flufenoxuron (μg per gram larva). n=30 for each injection dose.

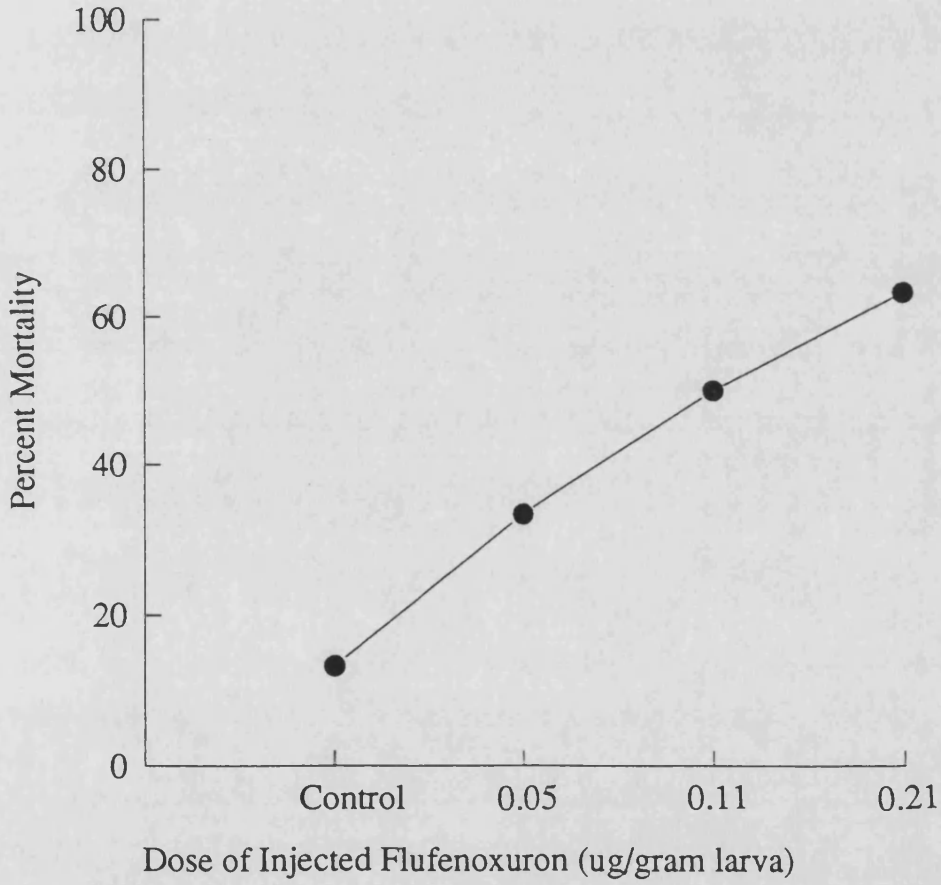

100
80
60
40
20
0
Percent Mortality
Control
0.05
0.11
0.21
Dose of Injected Flufenoxuron (ug/gram larva)

**Fig. 2.6.** Time course of incorporation of [$^{14}$C]-GlcNAc *in vitro*. Chitin synthesis was measured as incorporation by explanted proleg of [$^{14}$C]-N-acetyl-D-Glucosamine into KOH-soluble and -insoluble material in Day 0 5th stadium *Manduca sexta* larvae. Mean ± S.E.M. (n=5 per point). Points represent KOH-soluble (epidermal, ○) and KOH-insoluble (cuticular, ●) material.

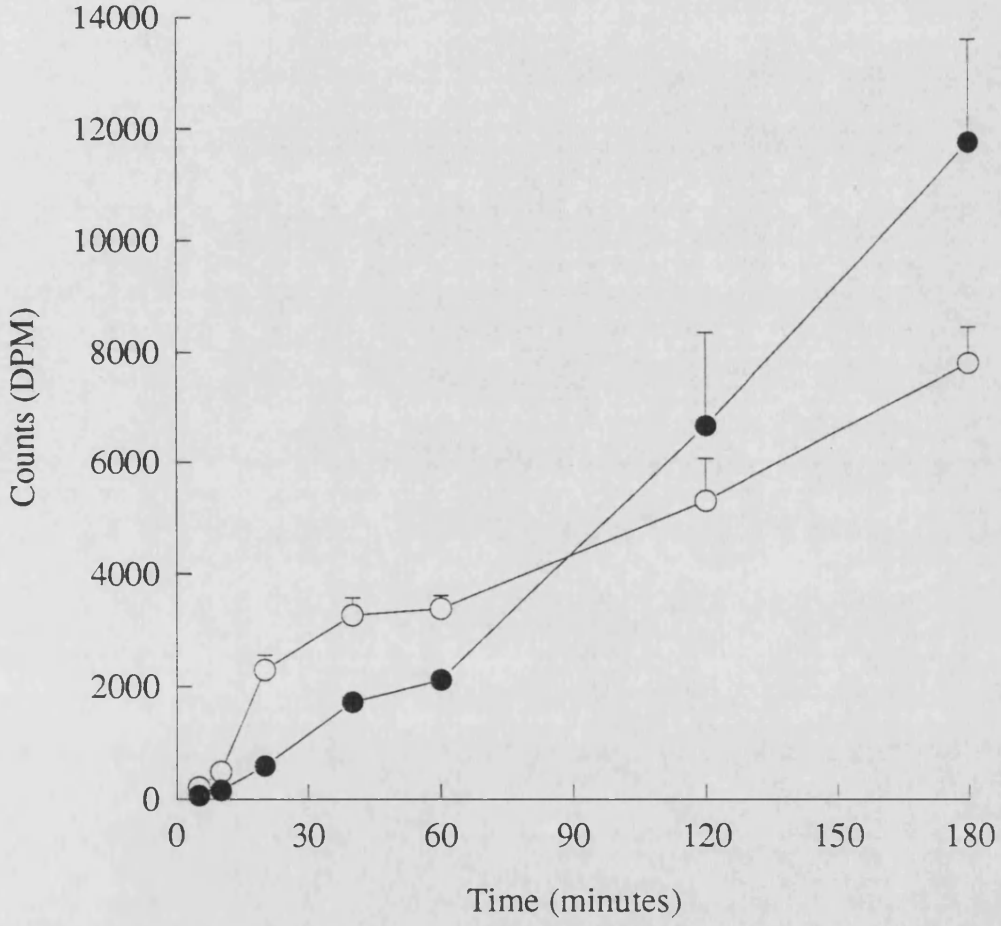

14000
12000
10000
8000
6000
4000
2000
0
Counts (DPM)
0
30
60
90
120
150
180
Time (minutes)

**Fig. 2.7.** Influence of Flufenoxuron on chitin synthesis in *M. sexta* prolegs. Points represent level of chitin synthesis expressed as a percentage of the control incorporation. --- corresponds to the $IC_{50}$ value. Details as for Fig. 2.6.

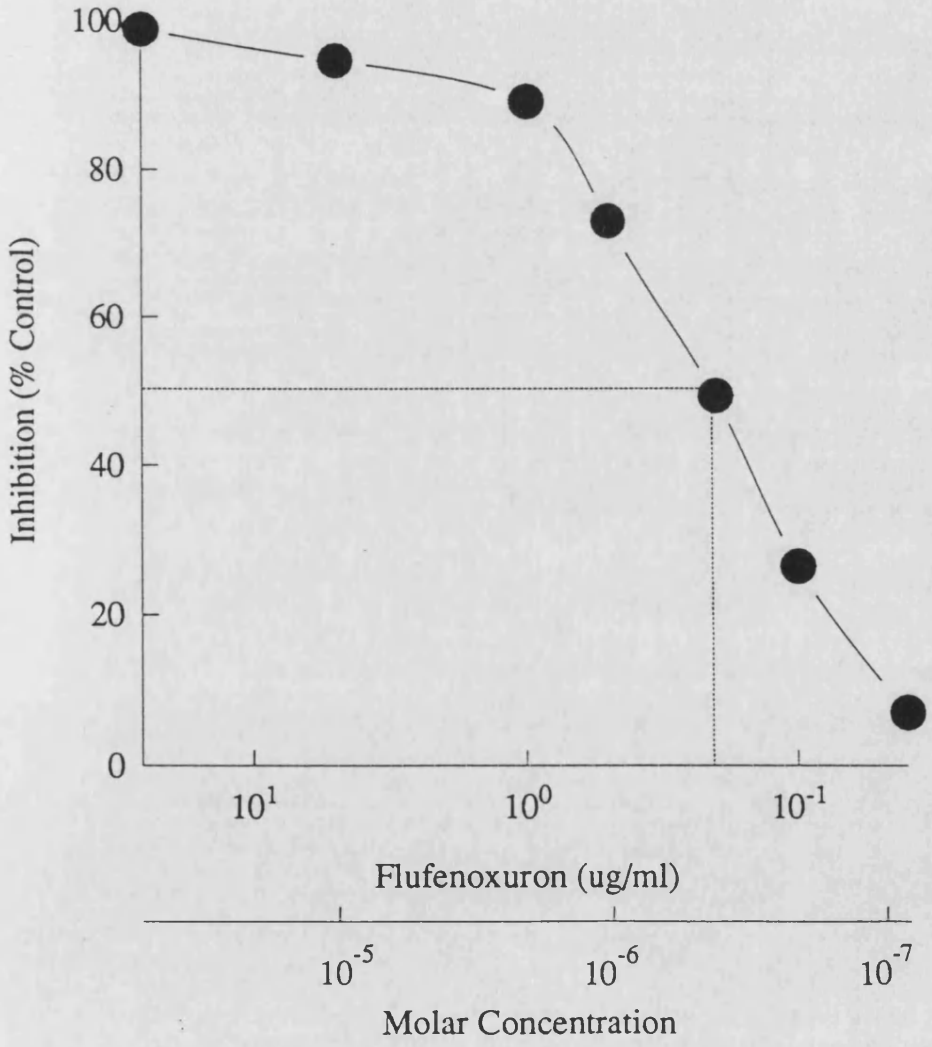

100
80
60
40
20
0
Inhibition (% Control)
$10^{1}$
$10^{0}$
$10^{-1}$
Flufenoxuron (ug/ml)
$10^{-5}$
$10^{-6}$
$10^{-7}$
Molar Concentration

**Table 2.1.**

The Effect of Injected flufenoxuron on fourth stadium *Manduca sexta* of different ages

| Age of stadium (days) | | 0 | 1 | 2 | 3 | 4(P) |
|---|---|---|---|---|---|---|
| Weight of stadium (gram) | | 0.3 | 0.6-0.8 | 1.10-1.20 | >1.30 | >1.30 |
| Mortality in a sample of 30 larvae, subject to: | | | | | | |
| | Symptom | | | | | |
| 0.43 µg | (Observation 1)[1] | 25* | 18* | 10* | 0* | 0* |
| | (Observation 2)[2] | 0* | 7* | 18 | 30* | 29* |
| | Total | 25 | 25 | 28 | 30 | 29 |
| 0.21 µg | (Observation 1) | 16* | 10 | 13 | 9 | 1* |
| | (Observation 2) | 0* | 5 | 9 | 11* | 9* |
| | Total | 16 | 15 | 22 | 20 | 10* |
| 0.11 µg | (Observation 1) | 15* | 3* | 9 | 7 | 6 |
| | (Observation 2 ) | 0* | 0* | 0* | 8* | 2 |
| | Total | 15* | 3* | 9 | 15* | 8 |
| Control | (Observation 1) | 2 | 2 | 3 | 3 | 0 |
| | (Observation 2) | 0 | 0 | 0 | 0 | 0 |
| | Total | 2 | 2 | 3 | 3 | 0 |

[1]Failure to grow in fifth stadium
[2]Died at ecdysis

(P) = Pharate fifth stadium larvae
*Significant result using chi-square test, $P<0.05$.

**Table 2.2.**

The Incorporation of [$^{14}$C]-Glucose into chitin and inhibition by injected flufenoxuron

| | Radioactive Counts |
|---|---|
| Control | 655.1 ± 126 |
| Flufenoxuron | 220.4 ± 34.4 |
| Percent Inhibition | 76.4 |

Day 0 fifth stadium *Manduca* larvae were given a single injection of flufenoxuron (100 ng) followed by an injection of [$^{14}$C]-Glucose (0.1 μCi, 230mCi mmol$^{-1}$) and maintained at the test temperature for 1 h. The amount of radioactivity incorporated at this time was determined with the proportion of inhibition by flufenoxuron expressed as a percentage of the control figure.

Counts measured as DPM mg$^{-1}$ dried cuticle.

Values expressed as means ± S.E.M. (n=10).

**Table 2.3.**

Uptake of different [$^{14}$C] precursors in *Manduca* saline

| | Time (min) | [$^{14}$C]-Glucose | [$^{14}$C]-GlcNAc |
|---|---|---|---|
| KOH Fraction | 60 | 4440.8 ± 992 | 3898.2 ± 306 |
| | 120 | 5846.1 ± 583 | 4163.4 ± 815 |
| Chitin Fraction | 60 | 262.7 ± 126 | 2277.6 ± 198 |
| | 120 | 952.8 ± 302 | 5787.0 ± 2104 |

Individual prolegs of Day 0 5th stadium *M. sexta* were incubated with 0.1µCi of either precursor.

Counts determined as mean ± S.E.M. (n=5).

**Table 2.4.**

(Expt.1)

Uptake of [$^{14}$C]-GlcNAc in different incubation media

| | Time (min) | Manduca saline | Grace's medium |
|---|---|---|---|
| KOH Fraction | 60 | 5205.0 ± 328 | 2790.4 ± 390 |
| | 180 | 4380.2 ± 502 | 3622.8 ± 247.3 |
| Chitin fraction | 60 | 1228.5 ± 237 | 997.0 ± 263 |
| | 180 | 1923.7 ± 523 | 1223.0 ± 494 |

(Expt 2)

| | Time (min) | Manduca saline | Grace's medium |
|---|---|---|---|
| KOH Fraction | 60 | 5153.5 ± 447 | 3646.4 ± 237 |
| | 180 | 8528.3 ± 791 | 6008.2 ± 513 |
| Chitin fraction | 60 | 2992.0 ± 389 | 3243.7 ± 534 |
| | 180 | 7500.0 ± 1612 | 5967.0 ± 1071 |

Individual prolegs of Day 0 5th stadium *M. sexta* were incubated with [$^{14}$C]-GlcNAc (0.1μCi).

Mean ± S.E.M. (n=5 per determination) in each experiment.

**Table 2.5.**

Uptake of [$^{14}$C]-GlcNAc in different *Manduca* saline preparations

| | Time (min) | Non-Aerated saline | Aerated saline |
|---|---|---|---|
| KOH Fraction | 60 | 4122 ± 364 | 3881 ± 228 |
| | 120 | 5575 ± 723 | 5644 ± 375 |
| Chitin Fraction | 60 | 1465 ± 174 | 2987 ± 357 |
| | 120 | 2314 ± 557 | 5659 ± 357 |

Individual prolegs of Day 0 5th stadium M. sexta were incubated with [$^{14}$C]-GlcNAc (0.1μCi).

Counts (DPM) expressed as mean ± S.E.M. (n=5 per determination).

**Table 2.6.**

(Expt. 1)

Incorporation of [$^{14}$C]-GlcNAc into Pharate 5th larvae

| Larval Stage | Fraction | 4th stadium cuticle attached | 4th stadium cuticle removed |
|---|---|---|---|
| Tanned Mandibles | KOH-soluble | 1472 ± 188 | 1856 ± 346 |
| | Chitin | 859 ± 295 | 2934 ± 832 |
| Untanned Mandibles | KOH-soluble | 1731 ± 343 | 2201 ± 468 |
| | Chitin | 1157 ± 321 | 3925 ± 522 |

(Expt. 2)

| Larval Stage | Fraction | 4th stadium cuticle attached | 4th stadium cuticle removed |
|---|---|---|---|
| Tanned Mandibles | KOH-soluble | 1812 ± 215 | 1691 ± 308 |
| | Chitin | 1290 ± 462 | 4306 ± 868 |
| Untanned Mandibles | KOH-soluble | 2767 ± 352 | 2289 ± 352 |
| | Chitin | 4170 ± 916 | 4892 ± 785 |

Individual prolegs of staged pharate 5th instar M. sexta larvae were incubated with [$^{14}$C]-GlcNAc (0.1μCi) both in the presence and absence of the overlying 4th stadium cuticle. Samples were left for 2 h at 25$^{o}$C before processing.

Mean ± S.E.M. (n=5 per determination) in each experiment.

# Chapter 3

# Short-Term exposure of *Manduca sexta* Cuticle to Flufenoxuron: An Ultrastructural and Cytochemical Study

## Introduction

The benzoylphenylureas inhibit the process of chitin synthesis (Mulder and Gijswijt, 1973) and thus the amount of chitin deposition into new cuticle is reduced (Hassan and Charnley, 1987) in insect integument. Their precise mode of action is unclear because chitin synthesis itself is not completely understood. However, formation of the proteinaceous matrix has been reported to be unaffected (Hunter and Vincent, 1974; Post *et al.*, 1974; Ker, 1978). Similarly, the amino acid composition of acylurea treated cuticle is unaltered (Ker, 1977).

Flufenoxuron is a novel acylurea compound because it demonstrates both acaricidal and larvicidal activity. Nevertheless as is argued elsewhere in this thesis its mode of action is probably similar to the rest of the acylurea group. Structural studies of flufenoxuron-treated *Spodoptera* cuticle have shown deposition of the endocuticle to be affected (Lee *et al.*, 1990).

Chitin synthesis can be affected within 15 min of application of the acylurea diflubenzuron (Deul *et al.*, 1978) in terms of inhibition of radiolabelled precursor incorporation although structural evidence of cuticular synthesis disruption is only apparent after longer exposure. Synthesis of chitin can recover from acylurea poisoning. Appropriate doses of acylureas, such as diflubenzuron, which are more readily metabolised than second generation acylureas like

flufenoxuron (Guyer and Neumann, 1988) can be shown to also disrupt cuticle (corresponding to insecticide potency) with the synthesis of apparently normal endocuticle re-established below the affected layers (Ker, 1978), presumably as the insecticide is removed by metabolism.

The aim of this study was to investigate the short-term effects of flufenoxuron on the cuticle using electron microscopy. The region of most interest was the epidermal/sub-cuticular region; because of the chronological order of cuticle deposition from the apical membrane of the epidermal cell, this area should be affected first. The lectin wheat germ agglutinin (WGA) was used to detect the presence of chitin. When linked to gold particles the WGA acts as an electron-dense specific cytochemical marker for trimers of N-acetylglucosamine (Goldstein *et al.*, 1975).

## Materials and Methods

*Insects*

Day 0 5th instar *Manduca sexta* larvae were taken from the rearing room and prolegs were removed (see Chp. 2) into *Manduca* saline. These were then incubated in Eppendorf tubes containing flufenoxuron (10 μM) in 100 μl saline (0.5% DMSO) for 15 min (corresponding to preincubation period in [$^{14}$C]-labelling expts. in Chp. 2) or 75 min (15min preincubation and 1 h main incubation). Control prolegs were treated identically with only DMSO. The dose of flufenoxuron corresponded to a concentration known to give >90% inhibition of [$^{14}$C]-GlcNAc incorporation *in vitro* (see Chp 2).

*Electron Microscopy*

Tissue was fixed in *Manduca* saline and 2.5% glutaraldehyde at 4°C for 24 h after being dissected into 2 x 2 mm fragments (approximately). Tissue was post-fixed in 1% osmium tetroxide, dehydrated through a graded series of alcohols and embedded in LR white resin (Agar Scientific, Essex, England).

Ultrathin sections were made on a Reichert Om U3 ultramicrotome, floated onto copper 200 grids, and stained with Uranyl Acetate (5 min in dark) and Lead Citrate (5 min) (Reynolds, 1963). Thin sections (gold, 90-150 nm) were examined and photographed in a JEOL JEM 1200EX transmission electron microscope operating at 80 or 100kV. The WGA-gold investigation used nickel 200 grids.

*Cytochemical detection of Chitin*

For detection of chitin, control and experimental cuticles were labelled with wheat germ agglutinin (WGA) coupled with 10nm gold particles (Polysciences 17639, Northampton, UK). Cut sections were first preincubated in 1% bovine serum albumin (BSA, Janssen, Beerse, Belgium) in phosphate buffer (100 mM, pH 7.2) for 20 min to reduce non-specific binding, and then labelled with WGA-gold (1:19 v/v phosphate buffer) for 1 h 30 min. After washing the grids in distilled water, the sections were viewed once stained (uranyl acetate and lead citrate).

The specificity of the WGA-gold technique for chitin was assessed by competitive inhibition with 50 mM N, N', N" triacetyl chitotriose (Sigma) on control sections. The inhibitor, in phosphate buffer, was simultaneously incubated with WGA-gold (1:19 v/v, 1 h 30 min).

## Results

*Ultrastructure of Fifth instar Control Cuticle*

Larval cuticle in Day 0 fifth stadium larvae of *M. sexta* (15 min, untreated) consists of an epicuticle, procuticle and subcuticle (Fig. 3.1) immediately above the apical microvilli of the epidermal cells (Fig. 3.2a), the nuclei of which lie in a basal position within the cell. The cuticle thickness of day 0 5th instar larvae measures about 17 μm (Fig. 3.1). Within the procuticle are seen layers of apparently parabolic arcs. This appearance is now well established to result from the helicidal orientation (see Chp.1. Fig. 1.2.) of the microfibril layers.

Microvilli were seen to protrude from the apical plasma membrane, as mentioned above. The apex of each microvillus had an electron-dense internal coating (Fig. 3.2a) known as a plasma membrane plaque (Locke and Huie, 1979). These plaques are believed to be the sites of formation of chitin microfibres. The crenellated appearance of the apical membrane was fairly regular in many of the photographs observed for this type of cuticle treatment (control) indicating uniform microvilli.

*Flufenoxuron-affected Cuticle*

After exposure to flufenoxuron for 75 min, the same length of time employed in the *in vitro* experiments that measured [$^{14}$C]-N-acetylglucosamine incorporation, no differences in the appearance of the epicuticle, procuticle or subcuticle were apparent when treated cuticle was compared to controls of the same incubation time. However, the apical microvilli of the epidermis appeared to be affected, with the microvilli appearing somewhat flattened (Figs. 3.2a and 3.2b). Chitin microfibres were clearly evident, closely approaching the apical epidermal surface, in both treated and control tissues. There was no sign of a

microfibre-free zone as has been reported in studies of acylurea action with longer incubation times.

*WGA-gold Chitin Localisation*

With the short-term nature of the study WGA-gold was used in an attempt to detect any changes in chitin synthesis that would otherwise be unobserved on a morphological basis. In Figs. 3.3(d-f) sections at various magnifications through the cuticle of an untreated control (75 min) are seen. Binding of WGA-gold indicates the presence of chitin within the lamellate cuticle. This binding was uniform over the entire procuticle as well as the subcuticle. Binding was not seen in the epidermal cells or microvilli, indicating that chitin is not present within the cells.

Triacetyl chitotriose (50 mM), an excellent competitive inhibitor for the WGA-chitin interaction (Goldstein *et al.*, 1975), displaced essentially all of the WGA gold particles from the cuticle sections (Fig. 3.3g), thus identifying WGA as chitin-specific.

In flufenoxuron-treated larvae (75 min), the pattern of WGA-gold binding was indistinguishable from that seen in untreated control cuticle (Fig. 3.3 a-c). The subcuticle, the most recently deposited layer of the cuticle, was well labelled at 75 min in both control and flufenoxuron-treated cuticle.

Again, however, these micrographs showed that the apical microvilli of the epidermal cells showed signs of disruption, these being either completely absent or markedly reduced. Interestingly, the reduced microvilli still had their membrane plaques which gave the membrane a 'dashed' appearance.

**Discussion**

The ultrastructural appearance of the cuticle and epidermis of the last stadium of *M. sexta* seen in this study (see Fig. 3.1.) agrees closely with that described previously by Wolfgang and Riddiford (1981), and is similar to that noted in other lepidopteran larvae (Filshie, 1982). The epidermal cells have a cuboidal appearance which changes to columnar at the end of the stadium (Wielgus and Gilbert, 1978). The apical microvilli of these epidermal cells have plasma membrane plaques (Fig 3.2a.) from which secretion of the fibrous cuticle arises (Locke, 1970; Locke and Huie, 1979). The epicuticle is corrugated, presumably allowing for the expansion that accompanies growth within the fifth stadium. At the level studied here it has a uniform dark-staining appearance. The procuticle is helicoidal throughout its thickness with an increase in lamellar thickness from the outside to the inside. The lamellar appearance of the procuticle reflects the orientation within it of chitin microfibres (Neville, 1975). The chitin microfibres are unstained, and appear light against the darker background of the protein matrix. "Cuticular columns", perhaps representing perpendicular microfibres are prominent in the procuticle.

Chitin biosynthesis occurs at a high rate during the early part of the larval 5th instar stadium (Mitsui *et al.*, 1980) and then declines. Larvae at this early stage in the stadium (0-24 h) were chosen so that any effects on chitin synthesis would be rapidly exhibited. The possibility of any age-related variability was minimised by using synchronous Gate 2 (Truman, 1972) larvae.

A number of other ultrastructural studies have shown that exposure of the integument to acylureas leads to the production of procuticle lacking the electron-lucent profiles of chitin microfibres. For example, Hassan and Charnley (1987) showed that *Manduca* treated with diflubenzuron *in vivo* for 24h deposited

amorphous procuticle lacking microfibres, while Lee *et al* (1990) found that *Spodoptera* treated with flufenoxuron *in vivo* produced a similar formless procuticle after 14 h. It has usually been assumed that the formless procuticle so produced lacks chitin because microfibres are absent (Ishaaya and Casida, 1974), although Hassan and Charnley (1987) pointed out that chitin might still be present, but in the form of disorganised short chains instead of helicoidally-oriented long microfibres. This possibility now seems unlikely since Lee *et al* (1990) found that formless procuticle produced by flufenoxuron treatment did not stain with WGA-gold, a specific stain for poly GlcNAc. The amorphous procuticle produced after acylurea treatment must therefore consist of matrix proteins alone. Several studies have produced evidence that synthesis of cuticle protein is unaffected by acylureas (Hunter and Vincent, 1974; Post *et al.*, 1975; Ker, 1977; Ker, 1978).

It is puzzling then, that the present study failed to find any structural disruption of the procuticle or subcuticle after 75min. It has been shown in Chp 2. that the dose of insecticide employed in these experiments would have been sufficient to inhibit GlcNAc incorporation into chitin by more than 90% in this time. Evidently, assuming that such inhibition occurred in the integument that was examined in the electron microscope, this inhibition does not result in noticeable alteration of cuticle structure within 75min. Three explanations seem possible. First, although the assembly of chitin microfibres may have been inhibited concurrently with the inhibition of GlcNAc incorporation, not enough chitin would have been synthesised in 75min to notice its absence in EM pictures. Second, it may be that inhibition of GlcNAc incorporation does not reflect precisely the inhibition of chitin synthesis. Perhaps incorporation of the tracer is inhibited more rapidly than is the assembly of chitin microfibres. This could be the case if the primary target of flufenoxuron were a transport process required to bring precursors to the site of assembly, as has been postulated by Mitsui *et al*

(1984, 1985). The third possibility is that under the *in vitro* conditions of the experiment, flufenoxuron inhibited not just chitin synthesis, but all cuticle components. If cuticle deposition halted altogether, then no region of chitinless cuticle would be seen. This is not what happens *in vivo* where diflubenzuron treatment of *Manduca* causes the deposition of an amorphous, apparently chitinless zone of procuticle (Hassan and Charnley, 1987), but Mitsui *et al* (1980) found that *in vitro* diflubenzuron completely inhibited all cuticle deposition in *Manduca* epidermal explants.

The WGA-Gold labelling confirmed the ultrastructural observations seen in unlabelled control and experimental larvae (75min). The lamellate cuticle in both groups showed a strong affinity for the cytochemical marker indicating that after this short exposure to flufenoxuron the disruption seen within the microvilli is not reflected in the apparently normal structure of the overlying subcuticle and procuticle. There was no difference between the distribution of the WGA-gold label between treated and control samples. In both cases, the lectin labelled only the procuticle and subcuticle, and the labelled structures extended all the way to the apical surface of the epidermal cells.

Despite the lack of obvious effects of flufenoxuron on the cuticle itself, there were changes in the appearance of the apical microvilli of the epidermal cells (Fig 3.2b and 3.3c). These were reduced or even entirely absent after 75min. Similar changes induced by diflubenzuron were noted by Binnington (1985) in blowfly larvae (*Lucilia cuprina*), and by Percy-Cunningham et al (1987) in *Choristoneura fumiferana*. In the latter case it was shown that although microvilli altered shape, the plasma membrane plaques persisted after treatment. With longer exposure to flufenoxuron (2 h) in *Spodoptera littoralis* (Lee *et al.*, 1990) vesicles containing dense material appeared to originate from the microvilli, which once extruded reverted to their normal shape.

In conclusion, brief exposure *in vitro* to the acylurea insecticide flufenoxuron, under conditions known to inhibit incorporation of labelled GlcNAc by more than 90%, had marked effects on the structure of the epidermal cells, but did not cause observable changes in the cuticle itself. The effect of flufenoxuron on the structure of the apical microvilli, may be evidence for an action of flufenoxuron which is primarily on cell membranes.

**Fig. 3.1.** Ultrastructure of the integument of Day 0 5th stadium *Manduca sexta* showing the characteristic features of untreated cuticle. pr, procuticle; ep, epicuticle; epc, epidermal cell; lm, lamellae; sc, subcuticle. Scale bar: 500 nm.

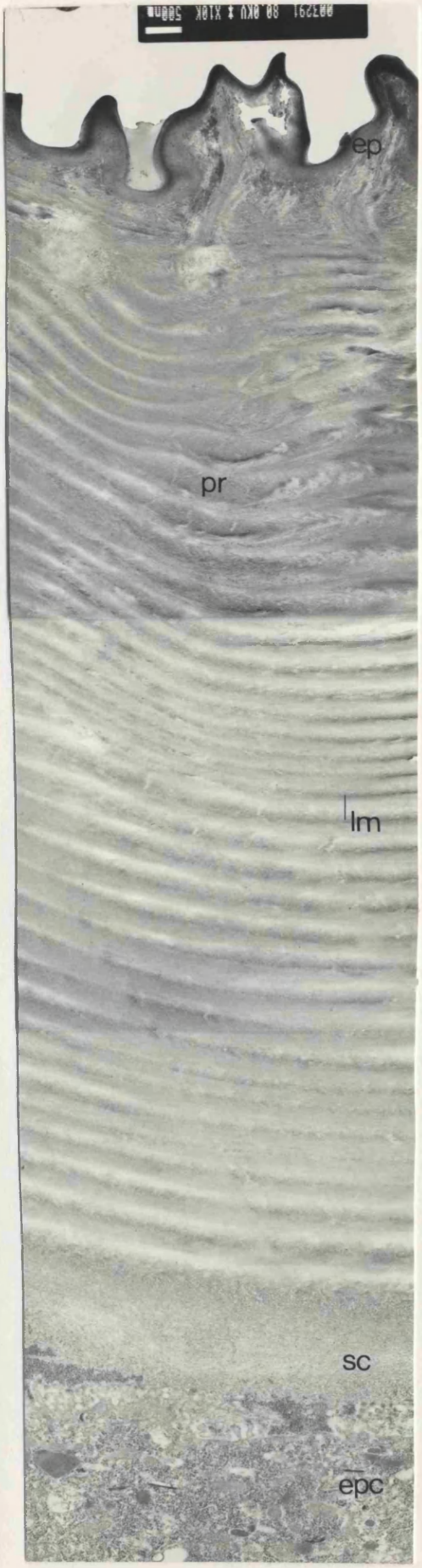
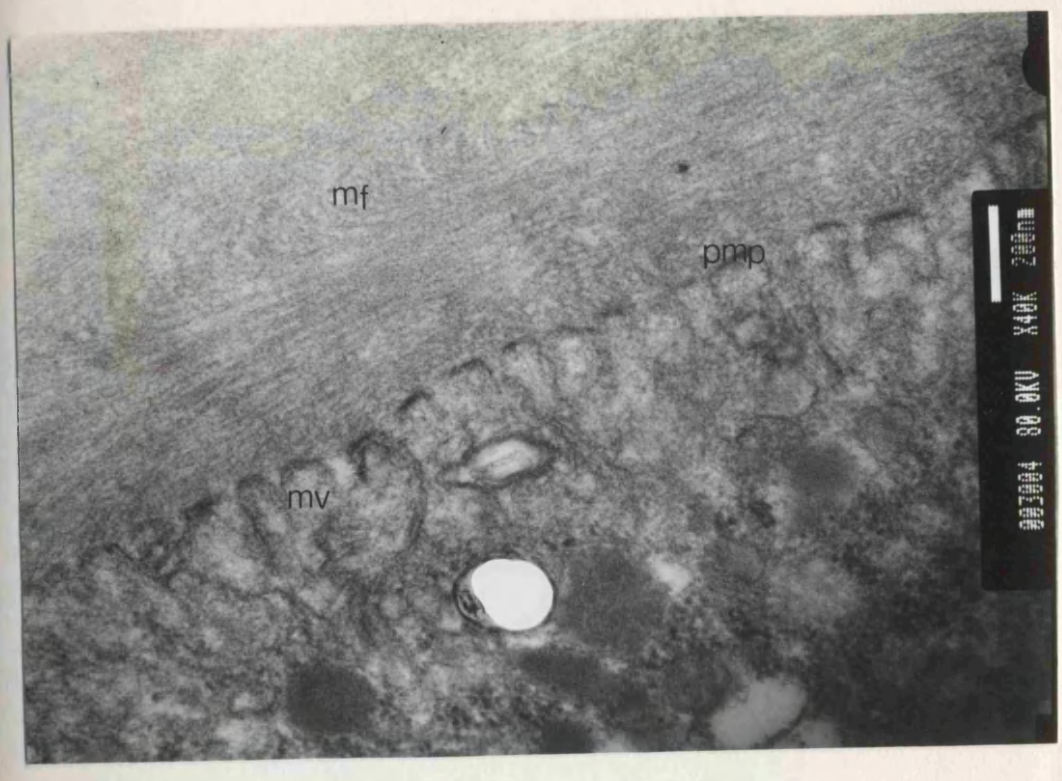

ep
pr
lm
sc
epc

**Fig. 3.2.** High power view of apical microvilli in (a) unteated and (b) flufenoxuron- treated (both 75 min) cuticle. mv, microvilli; pmp plasma membrane plaque; mf, chitin microfibres. Scale bar: 200 nm.

a




b

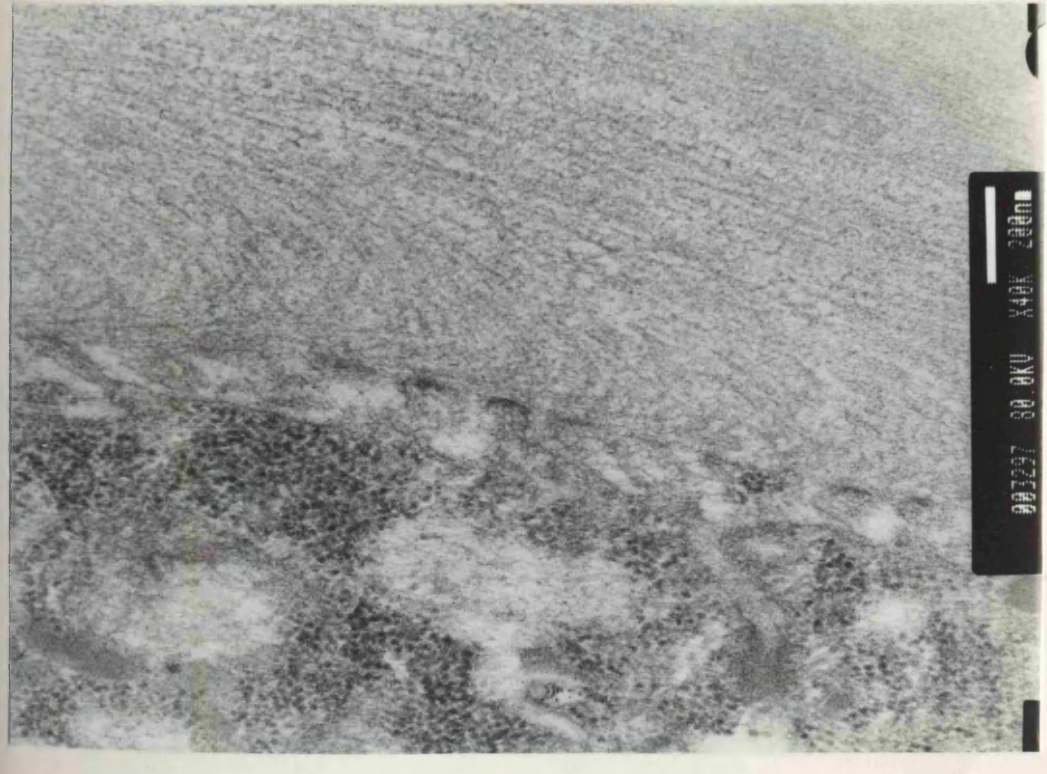
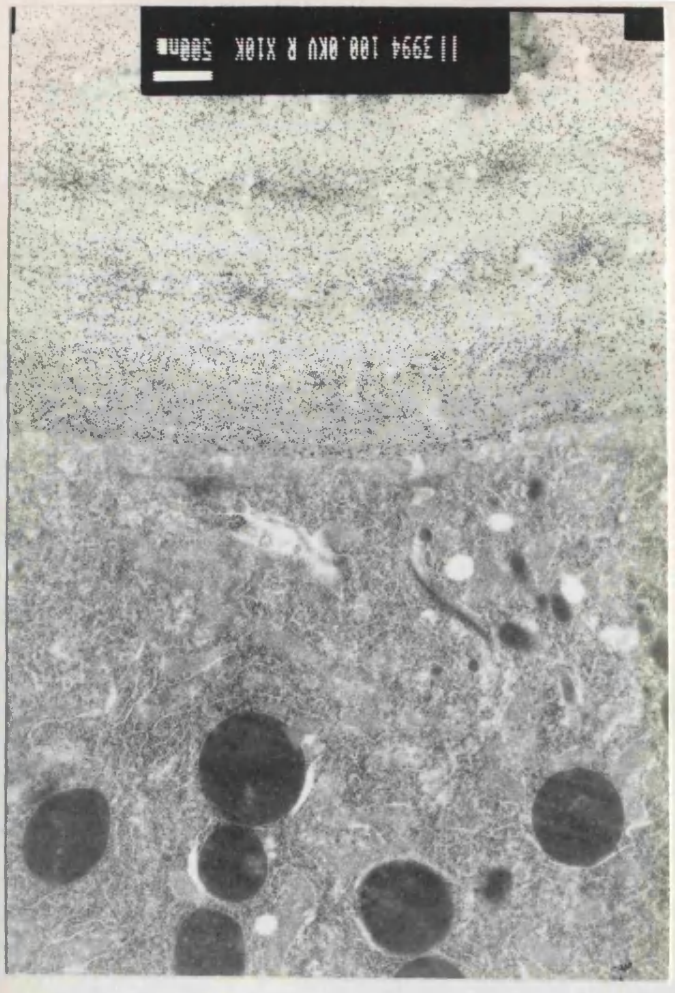


**Fig. 3.3.** Effect of exposure to flufenoxuron as revealed by WGA histochemistry. (a), (b), (c) Proleg integument treated *in vitro* with flufenoxuron (75 min), Stained with WGA gold. Scale bars: (a) 500 nm; (b) 200 nm; (c) 100 nm.

a




b

C

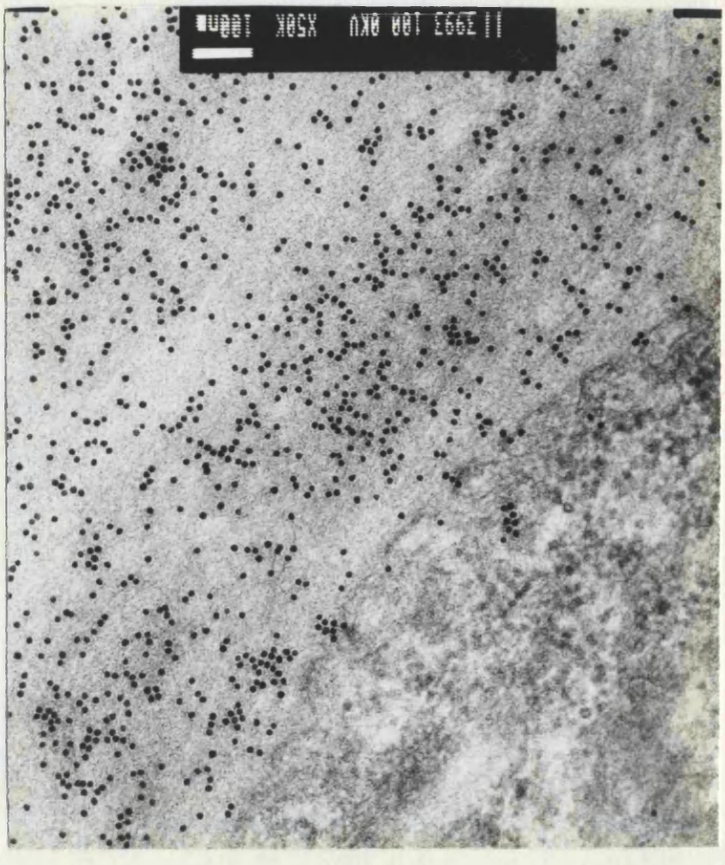
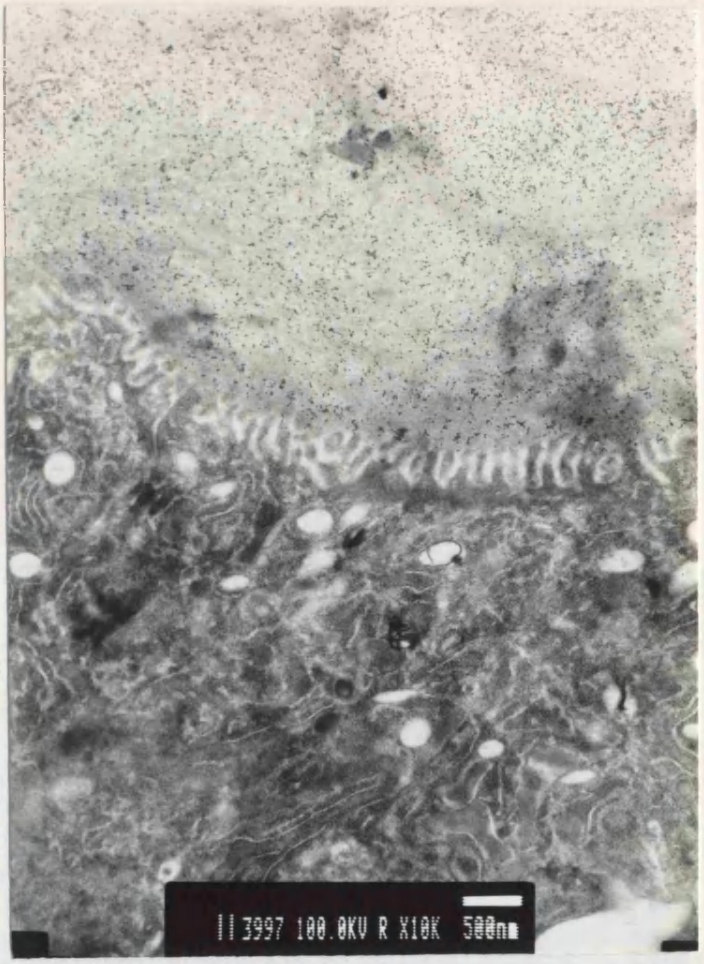
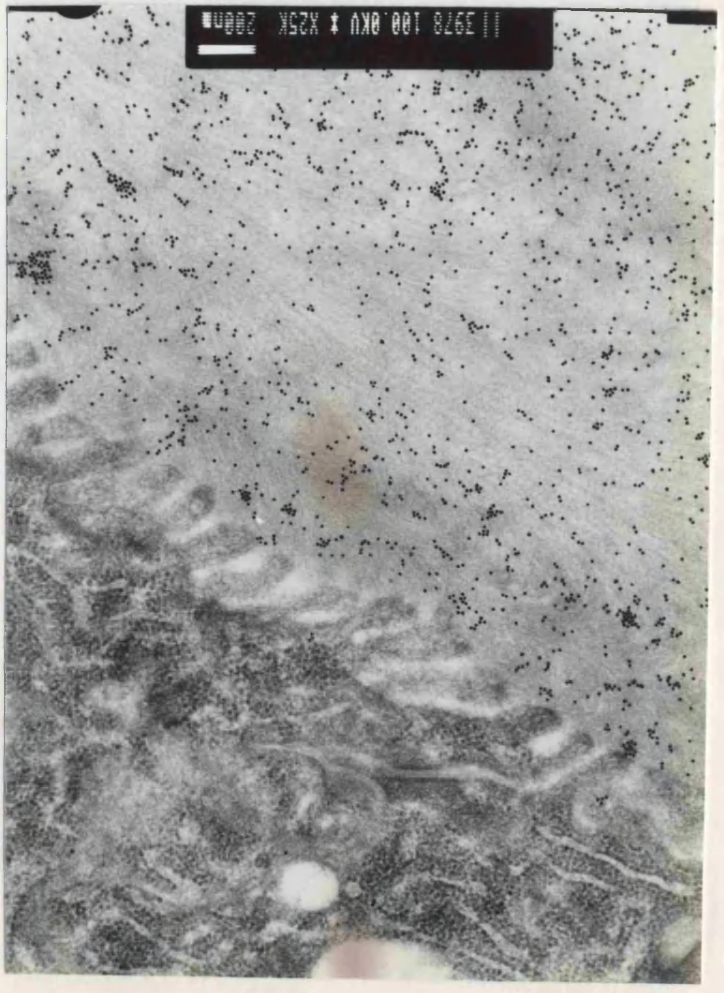


**Fig. 3.3.** (d), (e), (f) Control proleg integument (75 min), stained with WGA gold. Scale bars: (d) 500 nm; (e) 200 nm; (f) 100 nm.

d




e



f

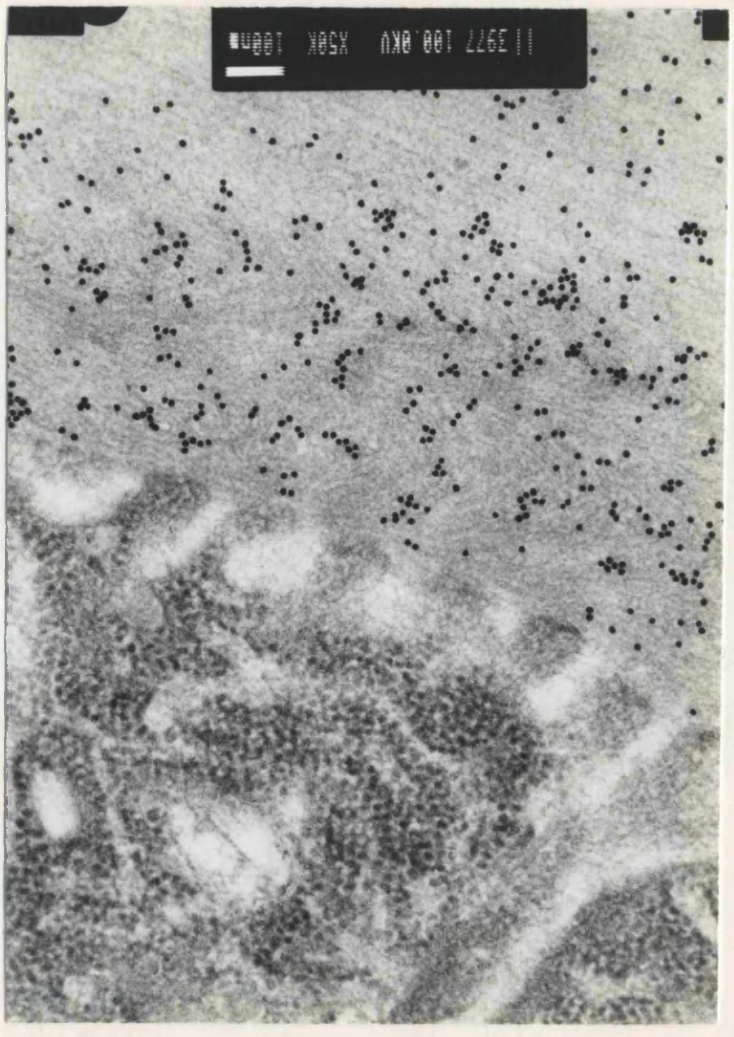
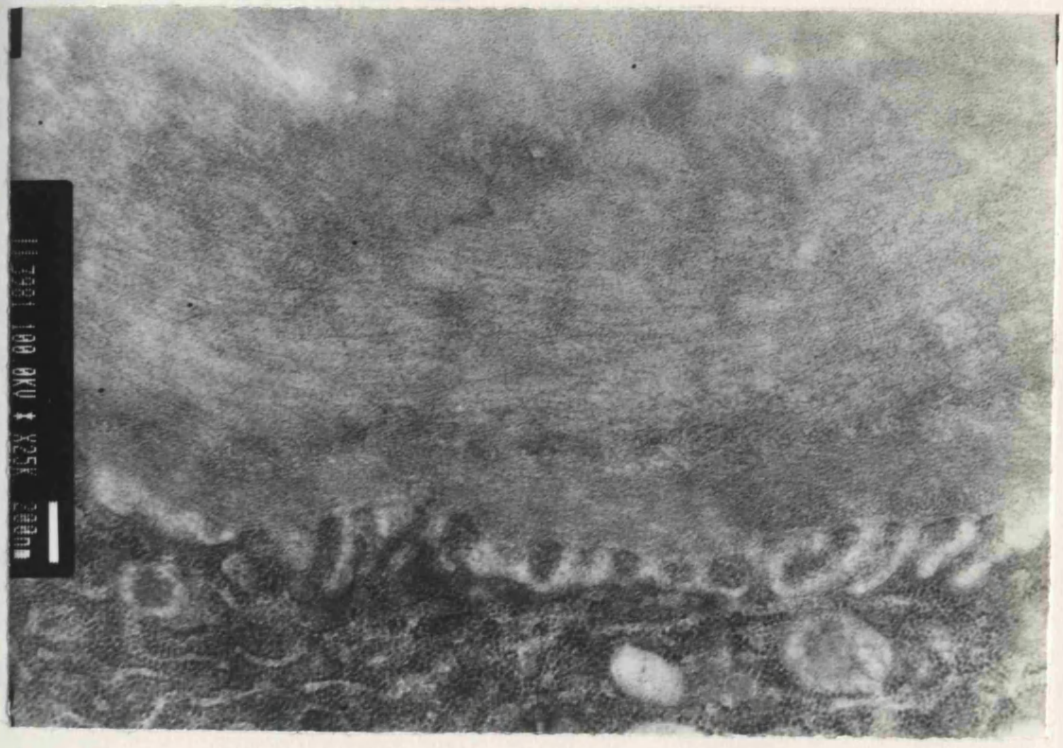


**Fig. 3.3.** (g) Control proleg integument (75 min), WGA gold staining displaced with the competitive inhibitor triacetyl chitotriose. Scale bar: (g) 200 nm.




g

# Chapter 4

# Temperature Effects on the Action of Acylurea Insecticides against *Manduca sexta* Larvae[1]

## Introduction

Acylurea (benzoylphenylurea) insecticides are agents with an insect growth regulator (IGR)-like action that results in delayed mortality of the treated insects, which usually die at the time of the next moult (Grosscurt and Jongsma, 1987; Reynolds, 1987). The cause of death is usually the rupture of the new cuticle during ecdysis, although in some cases the affected insects moult apparently successfully, but then fail to thrive and subsequently die (Neumann & Guyer, 1987). Sublethal effects can include a reduced rate of feeding and growth (Reed & Bass, 1979; Radwan *et al.*, 1986; Mitsui *et al.*, 1980).

Since the introduction of the first commercially useful acylurea agent, diflubenzuron (Mulder & Gijswijt, 1973), a number of other promising compounds have been developed. All appear to share the same mode of action, which is the inhibition of chitin synthesis (Reynolds, 1989). Failure to deposit chitin microfibrils in newly deposited cuticle renders it weak and ductile, especially at the time of the moult, accounting for the observed symptoms (Ker, 1977; Wolfgang & Riddiford, 1987).

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[1]This chapter is the same version as Chandler and Reynolds (in press)

Little is known about the effect of environmental temperature on acylurea action. We reasoned that the delay between exposure to the agent and subsequent death might cause the efficacy of these insecticides to be temperature dependent. We have investigated the problem using a range of acylurea compounds against a model Lepidopteran insect, the tobacco hornworm, *Manduca sexta*.

## Materials and Methods

### *Insects*

Tobacco hornworms, *Manduca sexta* (L) (Lepidoptera: Sphingidae), were reared on artificial diet according to standard methods (Bell & Joachim, 1976). The main culture was kept at 25°C under a long-day photoperiodic regime (LD, 17:7). Larvae were transferred to the test temperatures (20, 25 30 and 35°C) as required. These temperatures were maintained in cooled incubators each similarly fitted with lighting in phase with the original photoperiod of the stock rearing room.

### *Chemicals*

Insecticides were technical grade samples. Chlorfluazuron was a gift from Dr. R. Neumann, CIBA GEIGY AG, Basel, Switzerland; hexafluron was from P.K. Leonard, Dow Chemical Co., Letcombe Regis, UK; teflubenzuron was from Dr. W. Ost, Celamerck GmbH, Ingelheim am Rhein, FRG (now Shell Forschung); diflubenzuron, triflumuron and flufenoxuron were obtained from Shell Research Ltd, Sittingbourne, UK. [$^{14}$C]-flufenoxuron (0.11 μCi mmol$^{-1}$) was synthesised at Shell Research, while N-acetyl-D-[1-$^{14}$C]-glucosamine (58.7 mCi mmol$^{-1}$) was from Amersham. All other chemicals were of analytical grade from either Sigma or BDH.

*Insecticide treatments*

Where insecticides were incorporated into artificial diet this was done by mixing the appropriate amount of a 1 mg $ml^{-1}$ stock solution in acetone into the diet mix after it had cooled to 70°C. Made in this way, the content of acetone in the diet was never more than 0.1%. Controls were given diet containing the same amount of acetone as the experimental diet. For hatchlings, mortality and weight gain were assessed after 7 days continuous exposure to the insecticide-treated diet. For fourth stage larvae, insects were exposed to insecticide-treated diet only during that stage, and then transferred to untreated diet. In this case, mortality was assessed up to and including pupal ecdysis.

For injection, the stock solution of insecticide in acetone was rapidly mixed with an insect saline solution (Ephrussi & Beadle, 1936) to form a suspension. This was injected into the abdomen of the water anaesthetised insect from an SGE microsyringe equipped with a 28 swg needle. Controls received acetone/saline without insecticide. After injection the insects were briefly dipped in 70% ethanol to prevent infection. Test insects were fourth stage larvae less than 24 h after ecdysis. Mortality was assessed up to the onset of wandering.

*Uptake of [$^{14}C$]-flufenoxuron*

Day 0 fifth instar larvae were fed at 20, 25, 30 and 35°C on treated diet containing 0.03 ppm [$^{14}C$]-flufenoxuron for 24 h. At the end of this period, the larvae were killed by freezing and dissected into carcass and gut, and faeces were collected. The samples were lyophilised prior to combustion in a sample oxidiser (Packard); radioactivity was measured as $^{14}CO_2$. The amounts of radioactivity in the samples were expressed as the percentage of the total radioactivity (carcass, gut and faeces) recovered from each insect.

*Chitin synthesis in vitro*

Day 0 fifth stage gate 2 larvae (Truman, 1972) were anaesthetised in $CO_2$ and surface sterilised by swabbing with ethanol. The rear prolegs were removed and transferred to *Manduca* saline. Prolegs were first preincubated (15 min) at 20, 25, 30, or 35°C in 100 µl *Manduca* saline containing (control) 0.5% dimethylsulphoxide (DMSO), or (experimental) 0.41 µM flufenoxuron in 0.5% DMSO. Subsequently, the prolegs were transferred to identical solutions containing 17 µM [$^{14}$C]-N-acetylglucosamine (0.1µCi) for exactly 1 h. The incubation was stopped by transferring the prolegs to 0.3 ml 50% KOH at 100°C for 30 min (van Eck, 1979). Alkali-insoluble material was washed twice in distilled water and counted directly in 10 ml Optiphase Safe scintillant (LKB) in an LKB 1217 Rackbeta scintillation spectrometer. 87% of the radioactivity incorporated into KOH-insoluble material in this assay is chitin, as determined by solubilisation with fungal chitinase (D.R. Chandler, unpublished).

*Statistics*

Treatments at different temperatures were compared using a one way analysis of variance, deriving 95% confidence limits from pooled standard deviation using MINITAB. Values for $LC_{50}$ were estimated by probit analysis using GENSTAT.

## Results

*Temperature and acylurea action on Manduca larvae*

When newly hatched first stage *Manduca* larvae were exposed to discriminating doses of six different acylurea insecticides, it was found that in every case mortality increased progressively with temperature in the range 20°C to 35°C (Fig. 4.1). The concentrations of the different insecticides used differed

because some are more toxic to *Manduca* than others, but the actions of all the insecticides were affected by temperature in the same way.

It was observed for all the insecticides tested that the treated insects grew more slowly (gained less weight) than did controls, even where the treatment did not produce any mortality (Fig. 4.1). This sublethal effect, like that on mortality, was also positively correlated with temperature. Weight gain was less as a percentage of control at higher temperatures than at lower temperatures.

The situation was examined in more detail for flufenoxuron, where three different concentrations were tested. The conclusion was the same: that at any dose, both mortality and growth inhibition increased progressively with temperature (Fig. 4.2).

This effect of temperature was not confined to treatments of first stage larvae. When fourth stage larvae were given various concentrations of flufenoxuron in diet, we again found that mortality was greater at higher temperatures. Estimated $LC_{50}$ values were obtained for the insecticide's action at each temperature (Table 4.1). Flufenoxuron was significantly more toxic at 30 and 35°C than at 20 and 25°C.

*Temperature and acylurea uptake*

The effect of temperature on acylurea toxicity might have arisen from different rates of uptake of the insecticide. We investigated this problem in three ways.

First we administered single injections of a discriminating dose of flufenoxuron to newly ecdysed fourth stage larvae. Since this route of administration bypasses absorption from the gut, this experiment ought to show

whether the effect of temperature is on insecticide uptake. As is shown in Table 4.2, there was still a marked dependence of mortality on temperature, implying that temperature effects on flufenoxuron uptake are at most minor.

Second, we measured the amounts of food eaten by fourth stage larvae at each experimental temperature during the period in which they were exposed to insecticide, and thus computed the dose of insecticide to which the insects were effectively exposed. Table 4.3 shows that although the insects reared at higher temperatures eat more rapidly, they actually eat less food in total because the duration of the fourth stage is shorter at higher temperatures. Calculated values of insecticide intake from the $LC_{50}$ concentration in the diet show that the amount of flufenoxuron required to cause 50% mortality is less at higher temperatures than at low temperatures, whether this is computed in terms of intake per day or total intake.

In a third approach we measured the uptake of radiolabelled insecticide from the diet over a 24 h period. It was necessary to use fifth instar larvae (which are larger and eat more food) for this experiment because the specific activity of the available labelled insecticide (and therefore the amount of radioactivity accumulated) was low. The amounts of [$^{14}$C]-flufenoxuron taken up into the carcass or the gut by fifth instar larvae did not differ significantly ($P > 0.05$) between the four environmental temperatures when expressed as a percentage of the total amount of label eaten (Table 4.4). Thus the efficiency of insecticide uptake is not affected by temperature. Since the proportion of radioactivity present in the faeces did not vary between the different temperature regimes, it can be concluded that the extent of excretion of absorbed insecticide is also unaffected by temperature.

*Temperature and acylurea inhibition of chitin synthesis*

The effect of temperature on the toxicity of acylureas might be a consequence of a change in the intrinsic ability of the insecticides to interact with their primary target in the insect, the chitin synthetic processes of epidermal cells.

The rate of chitin synthesis was measured *in vitro* as the rate of incorporation of N-acetyl-D-[1-$^{14}$C]-glucosamine into KOH-insoluble material using explanted integument from the abdominal prolegs of day 0 fifth stage larvae. The effect of acute exposure to flufenoxuron on chitin synthesis was tested by exposure to the insecticide during a 15 min preincubation period before the addition of the labelled precursor to the medium, previously found to be sufficient to give maximal inhibition of synthesis.

Under these conditions, flufenoxuron inhibited chitin synthesis significantly less well at 20$^{o}$C than at the other temperatures tested (Fig. 4.3). However, there was no significant variation between the effectiveness of *in vitro* chitin synthesis inhibition at 25, 30 and 35$^{o}$C.

## Discussion

This study has shown that the action of a number of acylurea insecticides against the tobacco hornworm, *Manduca sexta*, is strongly affected by temperature. Mortality and sublethal inhibition of growth are both enhanced with increasing environmental temperature. We have investigated the case of flufenoxuron most thoroughly, but our findings indicate that all acylureas tested are affected by temperature in the same way.

The effects are substantial. In our experiments with single discriminating concentrations of insecticide given to newly hatched larvae in their food, the range of mortality experienced in the range 20°C to 35°C could vary from zero to 95% (eg 0.2 ppm flufenoxuron). The estimated $LC_{50}$ for flufenoxuron against fourth instar larvae varied by a factor of two.

The increased mortality and sublethal growth inhibition seen in insects exposed to acylureas at higher temperatures is certainly not due to an increased intake of insecticide treated food, whether reckoned in terms of the intake per day or over the feeding stage of an instar. The increased rate of food intake of insects at higher temperatures is insufficient to account for their increased sensitivity to the insecticide. In any case, the total amount of food eaten during the feeding stage of an instar is actually less at higher temperature than at lower ones; this is because the duration of the stage is shorter (Reynolds & Nottingham, 1985).

Experiments with single injected doses of insecticide that show a similar positive correlation of mortality with temperature suggest that the increased efficacy of acylurea at higher temperatures is not due to enhanced absorption from the gut. This conclusion is reinforced by direct measurement of the uptake of [$^{14}$C]-flufenoxuron from treated diet, which shows no significant effect of temperature on insecticide absorption.

We did not investigate the possibility that insecticide detoxication by enzymatic modification may be affected by temperature, but we regard this possibility as unlikely, since (a) this would have been likely to be reflected in increased excretion of radioactively labelled material (this did not occur), and (b) temperature affected in essentially the same way the efficacy of both diflubenzuron, which is subject to extensive metabolic detoxication by treated insects, and of chlorfluazuron, which is metabolised only rather slowly (Neumann

and Guyer, 1987). Nevertheless we are unable to exclude this possibility at present.

The intrinsic ability of flufenoxuron to inhibit chitin synthesis, thought to be the principal target site of acylurea insecticides (Reynolds, 1989), was clearly affected by temperature. Inhibition *in vitro* was significantly less at 20°C than at the three higher temperatures. However, there was no significant variation in inhibition between 25 and 35°C, suggesting that temperature effects on target site interactions are insufficient to account for all of the temperature dependence that we observed in experiments *in vivo*.

The remaining possibility is that insects kept at higher temperatures are intrinsically more susceptible to the inhibition of chitin synthesis, perhaps because their cuticle is qualitatively or quantitatively different from that of insects kept at lower temperature, or even because they are in some other way less "fit" (Reynolds and Nottingham, 1985), and therefore less able to withstand the weakening of their cuticle.

The only other study of which we are aware that reports the effect of temperature on acylurea action concerns the action of Dimilin (diflubenzuron) against *Simulium vittatum*, where a similar positive correlation of mortality with temperature was noted (Lacey & Mulla, 1978). The present study raises the possibility that such a relation may be general.

Temperature effects on insecticide efficacy are complex, and many interacting variables may be involved (Busvine, 1971). The unambiguous relation between temperature and acylurea toxicity to *M. sexta* exposed in this study seems worthy of note. The increased mortality due to acylurea treatment at high

temperature was consistently seen regardless of larval stage, and method of administration.

These effects occur within the range of temperatures that the insect is able to tolerate during larval life (Reynolds & Nottingham, 1985), and to which the insects are likely to be exposed in the field (Casey, 1977). It is not clear whether the temperature dependence of acylurea action is likely to be of significance in practical pest control; under field conditions, environmental temperature is almost certain to fluctuate considerably during each 24 h period, and perhaps to a lesser extent from day to day. Our findings suggest that it might at least be worth while to investigate the relationship of temperature to acylurea efficacy under real or simulated field conditions.

**Fig. 4.1.** The effects of temperature on lethal and sublethal effects of six acylurea insecticides on *M. sexta* larvae. Insecticides were incorporated into artificial diet at the concentrations shown. Newly hatched larvae were kept on treated or untreaed diet at the indicated temperatures. Mortality and growth in weight were assessed after 7 days. Mortality (○) is expressed as a percent of the total number of animals (20) in each group. Growth (●) is expressed as a percent of the control value at the same temperature.

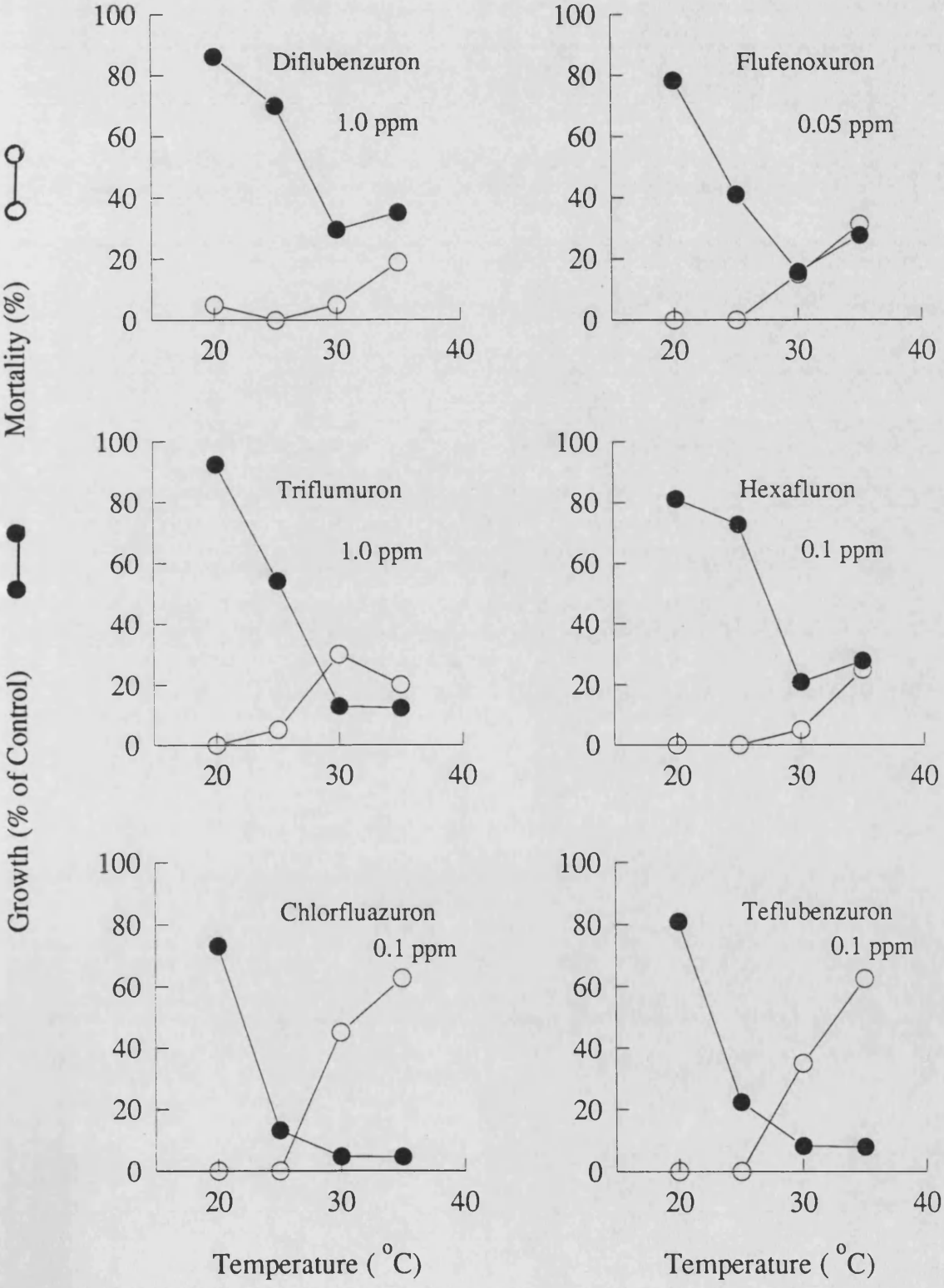
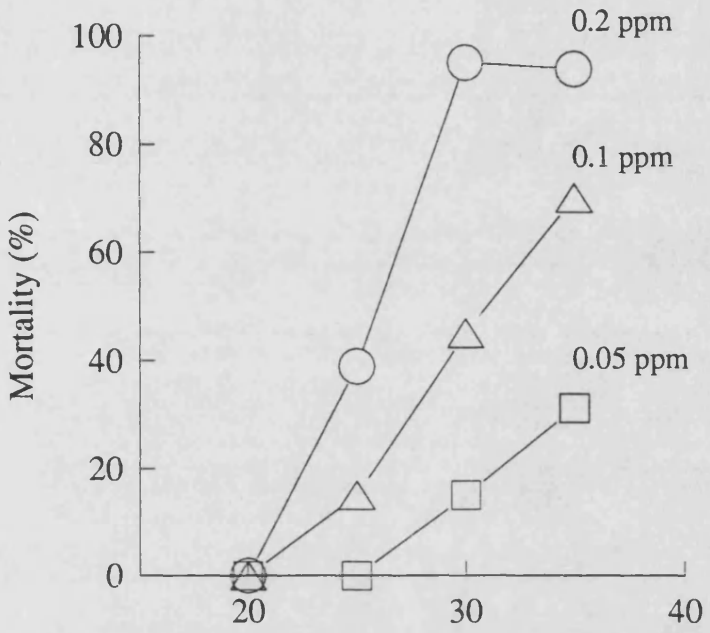

Mortality (%)
Growth (% of Control)
Diflubenzuron
1.0 ppm
Flufenoxuron
0.05 ppm
Triflumuron
1.0 ppm
Hexafluron
0.1 ppm
Chlorfluazuron
0.1 ppm
Teflubenzuron
0.1 ppm
100
80
60
40
20
0
20
30
40
Temperature ( °C)

**Fig. 4.2.** The effect of temperature on lethal and sublethal effects of three different concentrations of flufenoxuron on *M. sexta* larvae. Details as for Fig. 4.1.


Mortality (%)
100
80
60
40
20
0
20
30
40
0.2 ppm
0.1 ppm
0.05 ppm

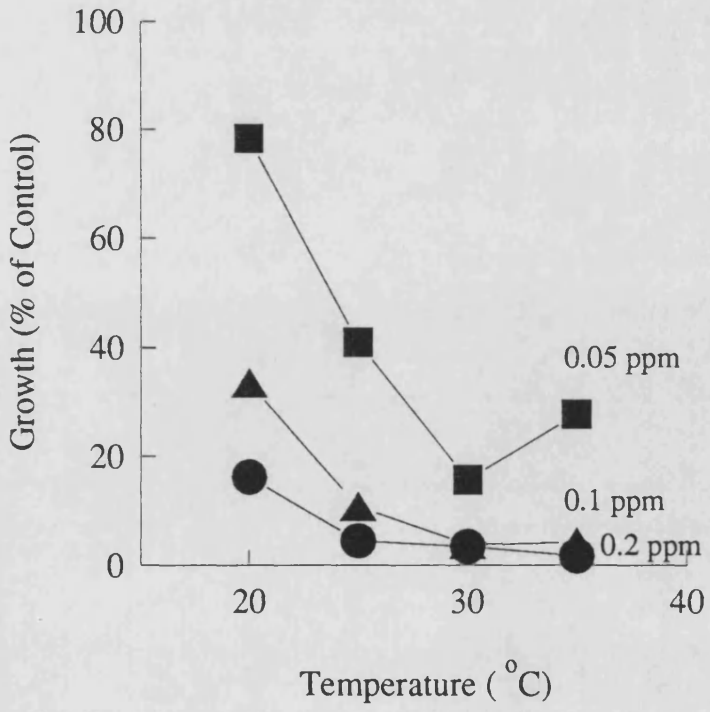

Growth (% of Control)
100
80
60
40
20
0
20
30
40
0.05 ppm
0.1 ppm
0.2 ppm
Temperature ( °C)

**Fig. 4.3.** The effect of temperature on the inhibition by flufenoxuron of chitin synthesis *in vitro*. Chitin synthesis was measured as incorporation by explanted proleg epidermis of [$^{14}$C]-N-acetyl-D-Glucosamine into KOH-insoluble material. Means $\pm$ S.E.M. (n = 4 per point). Significantly different values (ANOVA, $P<0.05$) are indicated by different letters.

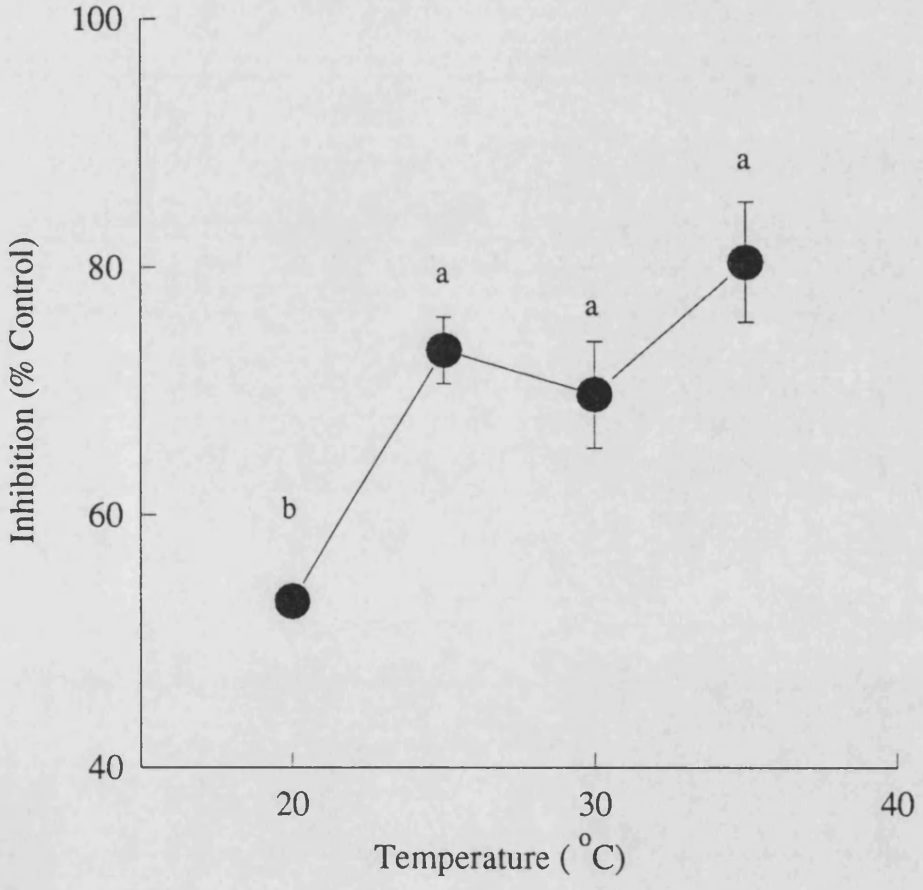

100
80
60
40
Inhibition (% Control)
a
a
a
b
20
30
40
Temperature ( °C)

**TABLE 4.1**

Toxicity of flufenoxuron for fourth stage *M.sexta* larvae.

| | 20°C | 25°C | 30°C | 35°C |
|---|---|---|---|---|
| $LC_{50}$ ± 95%c.i. | 0.041 ± 0.005 | 0.039 ± 0.006 | 0.021 ± 0.005 | 0.020 ± 0.005 |

Flufenoxuron given in the diet during the fourth stage only. Mortality assessed up to the time of wandering. $LC_{50}$ estimated from probit analysis using data from 9 different concentrations of insecticide between 0.005 and 0.20 ppm (n=10 for each concentration).

**TABLE 4.2**

Mortality following a single injection of flufenoxuron.

| Treatment | % Mortality | | | |
|---|---|---|---|---|
| | 20°C | 25°C | 30°C | 35°C |
| Control | 10 | 10 | 5 | 10 |
| Flufenoxuron | 20 | 35 | 95 | 90 |

A single injection of flufenoxuron (80 ng) was given to newly ecdysed fourth stage *M. sexta* larvae. Mortality was assessed up to the time of wandering. n=20 for each group.

**TABLE 4.3**

Intake of food and insecticide at different temperatures.

| | 20°C | 25°C | 30°C | 35°C |
|---|---|---|---|---|
| Diet eaten per day (g)[a] | 0.58±0.02 | 0.79±0.03 | 0.90±0.02 | 0.88±0.02 |
| Diet eaten in fourth stage (g)[a] | 2.60±0.07 | 2.37±0.08 | 2.26±0.09 | 2.20±0.05 |
| Estimated $LD_{50}$ Flufenoxuron intake per day (ng $d^{-1}$)[b] | 21 | 27 | 17 | 16 |
| Estimated $LD_{50}$ Flufenoxuron intake during 5th stage (ng)[b] | 94 | 82 | 42 | 39 |

[a] Means ± S.E.M. (n=10)

[b] Calculated from mean values of diet eaten (this table) and $LC_{50}$ (Table 1) and thus no statistical estimate of their accuracy can be made.

**TABLE 4.4**

Uptake of [$^{14}$C] flufenoxuron at different temperatures

| | 20°C | 25°C | 30°C | 35°C |
|---|---|---|---|---|
| % total radioactivity absorbed | 51.6±5.5 | 43.2±2.5 | 41.8±2.2 | 42.8±2.6 |

Day 0 fifth instar *Manduca* larvae were exposed to diet incorporating 0.03 ppm radiolabelled insecticide for 24 h at the test temperature. The proportion of the total radioactivity consumed that was incorporated into the carcass (ie the body minus the gut and its contents) was determined at the end of this time.

Means ± S.E.M. (n=5 for each temperature).

There is no significant difference between treatments (ANOVA, F = 1.72)

# Chapter 5

# Effects of the Acylurea Insecticide flufenoxuron on Cellular Nucleoside and Nucleotide levels in *Manduca sexta*

## Introduction

Chitin is one of a number of polysaccharides that are synthesised from sugar subunits coupled to the nucleotide uridine diphosphate (UDP). Chitin is synthesised from UDP-N-acetyl glucosamine and as the sugar subunits are attached to the growing chitin chain so the UDP is freed and rejoins the pyrimidine pool (Candy and Kilby, 1962). Inhibition of chitin synthesis by acylurea insecticides would be expected to cause changes in the levels of UDP and its metabolites. The prototype acylurea insecticide, diflubenzuron, has been shown to cause an accumulation of UDP-N-acetylglucosamine in treated insects (e.g. Gijswijt *et al.*, 1979; Hajjar and Casida, 1979). No information is available, however, for flufenoxuron.

The work described in this chapter attempts to follow changes in the cellular levels of uridine nucleotides following exposure to flufenoxuron using a thin layer chromatographic (TLC) technique. There are good reasons for examining the effects of acylureas on pyrimidine metabolism. There have been reports that acylurea treatment of insects inhibits DNA synthesis (DeLoach *et al.*, 1981; Soltani *et al.*, 1984) which indicates that acylureas might affect nucleotide metabolism. Additionally, there is a single report that one pyrimidine analogue, 6-azauridine, causes the accumulation in *Escherichia coli* of UDP-N-acetylglucosamine (Takagi and Otsuji, 1958). There is a structural analogy

between the structure of acylurea insecticides and the pyrimidine nucleus (Fig. 5.1.). It therefore seemed worthwhile to examine the effect on chitin synthesis of three well-known inhibitors of DNA synthesis that are analogues of uracil or uridine.

## Materials and Methods

### *Insects*

Tobacco Hornworms, *Manduca sexta* (L.) (Lepidoptera: Sphingidae) were reared on artificial diet under conditions previously outlined (Chp. 2, Materials and Methods). Newly moulted day 0 5th stadium larvae (Gate 2) were maintained at 25°C until required.

### *Chemicals*

The insecticide, flufenoxuron, was technical grade. This was a gift from Shell Research Ltd, Sittingbourne, UK. [5,6-$^{3}$H]-Uridine (48Ci mmol$^{-1}$) and N-acetyl-D-[1-$^{14}$C]-glucosamine (58.7mCi mmol$^{-1}$) were from Amersham. All other chemicals were of analytical grade either from Sigma or BDH.

### *Preparation of TLC plates*

Polyethyleneimine (PEI) cellulose F pre-coated plastic sheets (Merck, 20x20cm) were prepared before use (Randerath and Randerath, 1967) in order to remove UV-absorbing impurities. Each sheet was placed layer uppermost for 1 min in 10% NaCl and then allowed to dry for several hours. Sheets were then soaked in distilled water for 5 min and again allowed to dry. Finally, the cellulose layer was washed by ascending irrigation with distilled water in a glass TLC tank lined with Whatman 1 paper. When dry, the sheets were wrapped in foil and kept at -20°C.

*Incorporation of Radiochemicals into acid-soluble cell material by whole proleg explants.*

Rear prolegs of *M.sexta* were excised (as previously described in Chp. 2.) and placed into a *Manduca* saline-filled petri dish kept at room temperature (see Appendix A). When required, each proleg was individually transferred to an Eppendorf tube containing 100 µl of either control (4µCi [5,6-$^{3}$H]-Uridine, 0.5µCi [1-$^{14}$C] GlcNAc and 0.5 µl DMSO in *Manduca* saline) or test solution (identical but using 0.5 µl of 1 mg ml$^{-1}$ flufenoxuron in DMSO). After a 1 h or 5 h incubation at room temperature the tissue was removed and washed with 2x5 ml chilled saline within a Millipore filtration tower.

*Preparation of Acid-Soluble extracts*

Using a similar preparative technique to Plagemann *et al.* (1969) each proleg was placed in 0.2 ml of 0.5 N perchloric acid (PCA) and left to stand (0$^{o}$C, 30 min). The prolegs were treated with an MSE Soniprep 150 sonicator (9.5mm probe) for 30 seconds at 22 microns amplitude whilst kept on ice. Following this the Eppendorf tubes were centrifuged with an Eppendorf 5415 centrifuge (14 000x g, 5 min) and the supernatant was transferred to tubes containing 0.1 ml Tris-HCl (1 M, pH 7.4) and 15 µl 5 N KOH at 0$^{o}$C for 30 min. After centrifugation to remove the precipitate (14 000x g, 5 min), the corresponding supernatant samples (5 for each treatment) were pooled and put through a primed $C_{18}$ SepPak cartridge (Waters). This was washed with 3 ml 0.1% Trifluoroacetic Acid (TFA) and radiochemicals were eluted with 4 ml 80% Acetonitrile (AcN) and 0.1% TFA. Each total sample was then dried down in a rotary evaporator (Savant Speed Vac Concentrator).

*Chromatography of Radiolabelled Uridine-derived Nucleotides and Nucleosides*

Samples were redissolved in 15 µl 50% ethanol and were applied onto the previously prepared TLC plate. The plate was developed by ascending chromatography at room temperature for 20 min in 1 M acetic acid before transferring to 9 volumes 1 M acetic acid and 1 volume 3 M lithium chloride for 1 h (Randerath and Randerath, 1965).

All experimental samples were co-chromatographed with a 10 µl cocktail of nucleoside and nucleotide standards (UMP, UDP, UTP, UDP-GlcNAc, Uracil and Uridine) each at 10 mM. After development, the paper was dried and examined under shortwave ultraviolet light in a dark room to determine $R_f$ values of the standards..

TLC Chromatograms were divided vertically into the experimental lanes before being cut, starting at the base line, into 4mm segments at right angles to the direction of migration. Each segment was suspended in 0.5 ml PCA in a scintillation vial and heated to 80°C with the lid on for 30 min. Once cool, 5 ml of scintillation fluid was added and the tubes counted for radioactivity on a dual $^{3}H$-$^{14}C$ parameter programme.

*Effect of DNA synthesis inhibitors on incorporation of [$^{14}C$]-GlcNAc into Manduca cuticle*

Prolegs from Day 0 5th stadium *Manduca sexta* larvae were removed and incubated in 100 µl *Manduca* saline containing [$^{14}C$]-GlcNAc (0.1µCi). Experimental prolegs were incubated in a similar manner with saline also containing 6-Azauracil, 5-Fluorouracil or 6-Azauridine (all Sigma) at 0.41 µM ($IC_{50}$ for chitin synthesis with flufenoxuron) and also at the rather high concentration of 50 $\mu$g $ml^{-1}$. After 1 h prolegs were processed to measure radioactive incorporation into chitin (see Chp.2., Materials and Methods).

## Results

*Time course of [$^3$H]-Uridine uptake*

The exact time course of uptake was rather variable between experiments but a representative result is shown in Fig. 5.2. Uptake is time dependent up to 2 h, when the acid-soluble counts stabilise around 22 000 cpm.

*$R_f$ values of Nucleosides, Nucleotide sugars and Bases*

Table 5.1. shows the $R_f$ values for the series of uridine phosphorylated standards and uracil. The use of the aqueous solvent solution meant that the phosphate-bearing nucleotides were separated according to the number of phosphate groups, and hence net charge. UMP migrated furthest of the nucelotides, with UTP migrating the least. Uridine and uracil migrated much further. These compounds, with $R_f$ values >80, tended to form elongated spots because they migrated close to the solvent front.

The 1 h control profiles showing distribution of [$^3$H]-Uridine and [$^{14}$C]-GlcNAc, either in the native or phosphorylated form in *Manduca* proleg epidermis, are shown in Fig. 5.3.a. The radioactivity for [$^3$H]-labelled compounds peaks sharply at strip #23 and then slowly tails off. This probably represents free [$^3$H]-uridine and/or uracil. Radioactivity derived from the [$^{14}$C]-GlcNAc isotope shows a double peak with peaks occuring in #23 and #26. These [$^{14}$C]-labelled metabolites were not identified.

The 1 h insecticide-treated show a similar pattern of radiolabelling distribution (Fig. 5.3.b.). Again the [$^3$H] counts rise rapidly at #25 and tail off further up the TLC plate, representing labelled uridine and/or uracil. [$^{14}$C]-labelled products also occur in the same region, but with some counts displaced toward the solvent front. There is a slight rise in [$^{14}$C]-GlcNAc-derived counts on

strips #10 - #11 indicating a possible build-up of [$^{14}$C] in a more polar metabolite than the main peak. This could be UDP-GlcNAc from its $R_f$. A slight increase in tritiated counts could also be seen in strips#1-#9.

Figure 5.4.a. shows the distribution of [$^{3}$H] and [$^{14}$C] radioactivity in control epidermis after 5 h. The counts from [$^{3}$H]-Uridine show a broad band of low activity counts in the first 12 strip bands and the much higher activity broad band peaking at strip #24 as seen for Fig. 5.3.a. [$^{14}$C] distribution is again found mainly between strips #22 - #36.

The buildup after 5 h of radioactivity derived from [$^{3}$H]-Uridine in strips #1 - #10 is more pronounced after 5 h (Fig. 5.4.b.) in the insecticide-treated prolegs indicating that flufenoxuron causes accumulation of material probably corresponding to uridine di- and/or triphosphates, as determined by their $R_f$ values (see Table 5.1.). Strip No. #13 shows high counts of both [$^{14}$C]-GlcNAc and [$^{3}$H]-Uridine derived radioactivity that is not seen in any of the other treatments. The fact that this peak is doubly-labelled and its $R_f$ indicate that this must represent UDP-GlcNAc, the precursor to chitin. The uncharged radiolabelled parent compounds again show a wide peak near the solvent front.

*The effect on chitin synthesis by inhibitors of DNA synthesis*

The chitin synthesis inhibiting activity of the DNA synthesis inhibitors 6-azauracil, 5-fluoruracil and 6-azauridine to new cuticle development *in vitro* is shown in Table 5.2. It is clear that these compounds do not exert an inhibitory effect either at $IC_{50}$ concentration for flufenoxuron or at the high concentration of 50 $\mu g\ ml^{-1}$.

## Discussion

Initially it was necessary to observe the kinetics of uptake of [$^3$H]-uridine into the acid-soluble cellular fraction to establish appropriate conditions for subsequent labelling experiments. Although the results showed a time-dependent build-up of [$^3$H] counts the exact time course was rather variable between experiments. In general, what was observed was a rapid uptake of [$^3$H]-Uridine amounting to several thousand counts within a few minutes which then stabilised after 2 hours. This result of course takes no account of metabolic conversion of the labelled uridine after uptake into the cells. This initial, rapid uptake of [$^3$H]-Uridine, is also seen in mammalian cells (Plagemann *et al.*, 1969; reviewed by Plagemann and Wolhueter, 1980).

TLC analysis of control extracts showed that the main acid-soluble [$^3$H]-labelled compounds in the cell extracts probably correspond to free [$^3$H]-uridine or [$^3$H]-uracil. It is not possible to be more positive in this identification because of the elongated nature of the spots and variability between individual chromatograms. The identity of the [$^{14}$C]-labelled compounds in the same extracts was not investigated but the main [$^{14}$C] counts also elute close to the solvent front, similarly occurring in rather elongated spots. The colocalisation of the [$^{14}$C]-label with the [$^3$H]-label is probably coincidental. It is suggested that this [$^{14}$C]-label corresponds to unchanged GlcNAc, which is uncharged and would be expected to migrate close to the solvent front.

After 1 h exposure to flufenoxuron the main effect seen is a reduction in the amount of [$^3$H]-label relative to the amount of [$^{14}$C]. This is due to the apparent reduction in the amount of labelled nucleoside, while the labelled sugar remains constant. After 5 h, the amounts of [$^3$H]-labelled nucleoside in treated and control extracts are similar, while the amount of [$^{14}$C]-label, presumably

unchanged GlcNAc, is increased in the treated extracts compared with control. The significance of the changes is uncertain. As previously mentioned, the time course of uptake of [$^{3}$H]-uridine was somewhat variable between experiments. Klitschka *et al.* (1986) reported that diflubenzuron inhibited uridine uptake by cultured Lepidopteran cells. They found that high concentrations (> 50 μM) were required. Uridine incorporation reduced, as was incorporation of adenosine, cytosine and guanine. They also found that a derivative of the triazine insect growth regulator cyromazine (Vetrazin), was the most potent inhibitor. Cyromazine is noted to have no effect on chitin synthesis (Turnbull and Howells, 1982) although cuticle synthesis is affected (Binnington, 1985).

Of more significance is the observation that flufenoxuron treatment causes clearly enhanced conversion of [$^{3}$H]-Uridine into UDP and/or UTP after 5 h, with a smaller but significant enhancement after only 1 h. Similarly, both [$^{3}$H]-Uridine and [$^{14}$C]-GlcNAc are converted to double-labelled UDP-GlcNAc in increased amounts after flufenoxuron treatment, the change being very clear after 5 h, but evident after only 1 h. A build up of UDP-GlcNAc following treatment with the prototype acylurea diflubenzuron has been reported previously (Hajjar and Casida, 1978; van Eck, 1979, Turnbull and Howells, 1982). The present establishes that this also occurs with flufenoxuron, reinforcing the evidence presented in other chapters that this compound shares the same mode of action as diflubenzuron.

This is the first time that a double labelling technique has been used to show this accumulation of UDP-GlcNAc. The method allows the demonstration of UDP-GlcNAc build-up after only 1 h. Previously, much longer treatment times (the cell-line experiment of Klitschka *et al.* (1986) required a minimum of 10 days *in vitro* incubation.) were required.

The 5 h-treated samples showed a build-up of polar [$^3$H]-derivatives that probably correspond to UDP and UTP. A build-up of UMP was not observed. This accumulation of uridine nucleotides may suggest a "knock-on" effect from the observed build-up of UDP-GlcNAc through feedback inhibition consequent upon inhibition of chitin synthesis. An alternative interpretation would be that this accumulation of UDP/UTP is a primary feature of acylurea action, and that the inhibition of chitin synthesis is secondary to it. This is a possibility since insect chitin synthase is known to be inhibited by high concentrations of UDP (Cohen and Casida, 1982).

There are several reports that acylurea treatment can inhibit DNA synthesis in insects (Soltani *et al.*, 1980; Mayer, 1980). Therefore it was relevant to ask if this is a primary effect of the insecticides, or secondary. One test of this would be to determine if artificial inhibition of DNA synthesis in turn inhibits chitin synthesis. The anticancer agents 5-Fluorouracil, 6-Azauracil and 6-Azauridine are analogues of uracil and uridine whose metabolites inhibit DNA and RNA synthesis. 6-Azauridine was also noted to cause a 5 to 6-fold build-up of UDP-N-acetyl-Glucosamine in acid-soluble fractions of *Escherichia coli* (Takagi and Otsuji, 1958); an essential metabolite for formation of the bacterial cell wall. As the results in Table 5.2 show, this is not so. It is concluded that inhibition of DNA synthesis is not a cause of chitin synthesis inhibition. Rather, the reverse is likely. All published reports of DNA synthesis inhibition by acylureas required long (days) periods of exposure to the insecticide. After this length of time, larval development is generally disrupted.

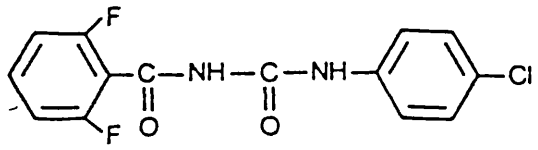
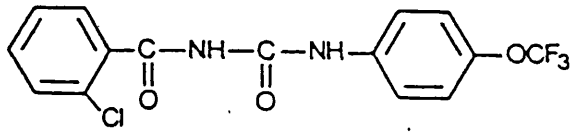
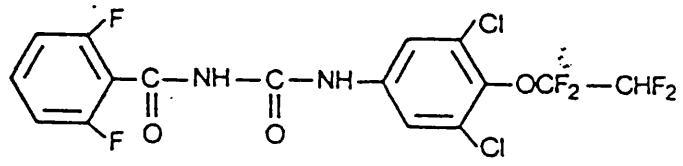
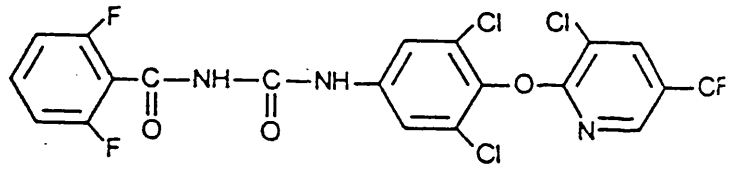
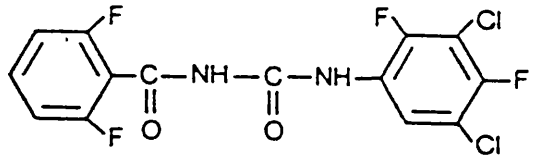
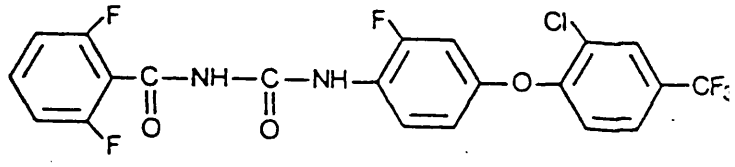
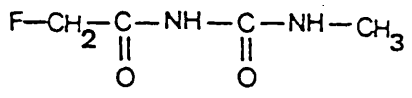
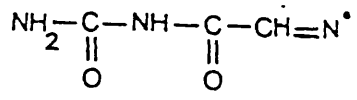
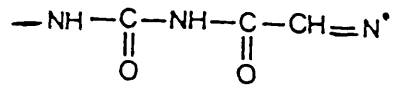
| | Chemical Structure |
|---|---|
| **Diflubenzuron** |  |
| **Triflumuron** |  |
| **Hexafluoron** |  |
| **Chlorfluazuron** |  |
| **Teflubenzuron** |  |
| **Flufenoxuron** |  |
| 5-Fluorouracil |  |
| 6-Azauracil |  |
| 6-Azauridine |  |

**Fig. 5.1.** Structural analogy between the pyrimidine nucleus of uracil/uridine derivatives and 6 acylurea insecticides.

**Fig. 5.2.** Time course of incorporation of [$^{3}$H]-Uridine *in vitro*. Uptake was measured as incorporation by explanted proleg of [5,6-$^{3}$H]-Uridine into acid-soluble extracts in Day 0 5th stadium *Manduca sexta* larvae. Mean ± S.E.M. (n=5 per point).

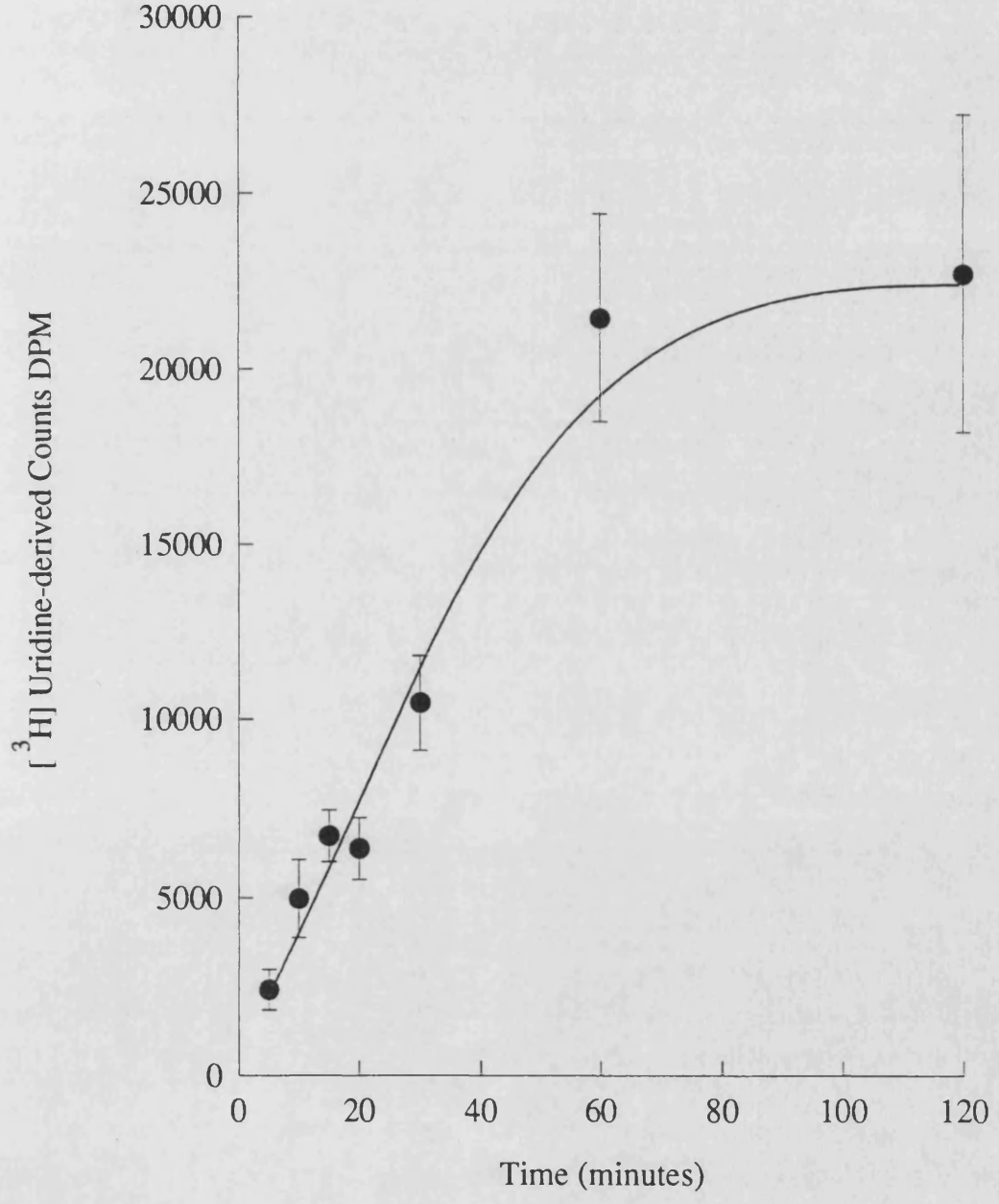
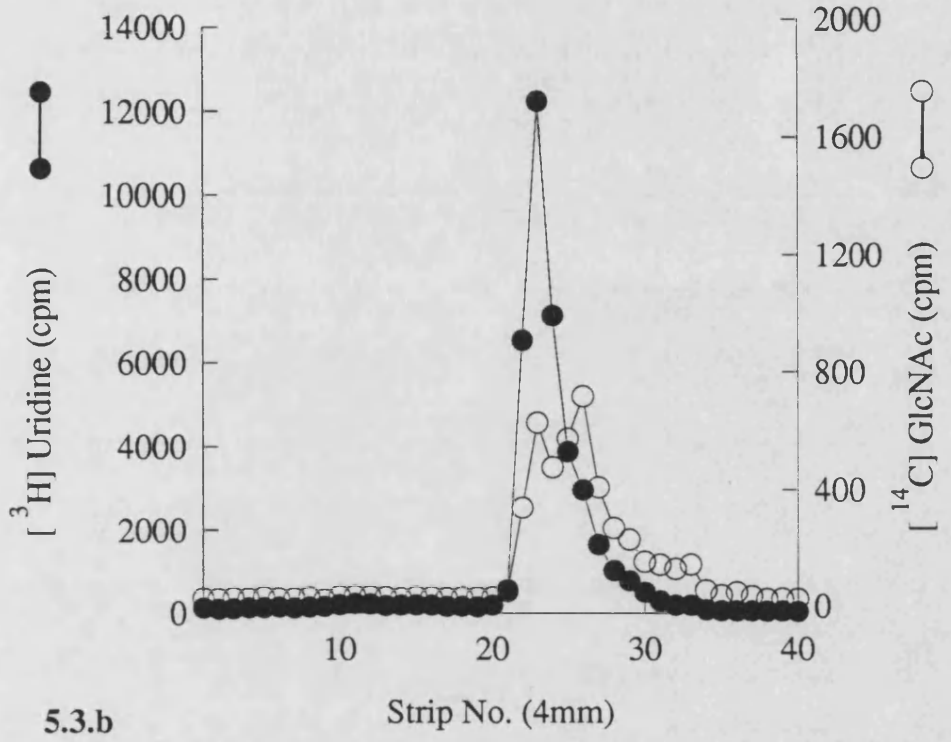

30000
25000
20000
15000
10000
5000
0
0
20
40
60
80
100
120
[ 3H] Uridine-derived Counts DPM
Time (minutes)

**Fig. 5.3.** TLC profile of radioactivity derived from [$^{3}$H]-Uridine and [$^{14}$C]-N-acetylglucosamine incorporation into *M. sexta* epidermal tissue after 1 h incubation following perchloric acid extraction. **a**) Control (DMSO solvent only). **b**) Treated with flufenoxuron (0.01 mM). Radioactive counts (cpm) in each 4 mm strip are the pooled result of 5 explanted prolegs. Inset shows detail of cpm in the first 20 strips from baseline.

**5.3.a**


14000
12000
10000
8000
6000
4000
2000
0
2000
1600
1200
800
400
0
10
20
30
40
[ 3H] Uridine (cpm)
[ 14C] GlcNAc (cpm)
Strip No. (4mm)

**5.3.b**

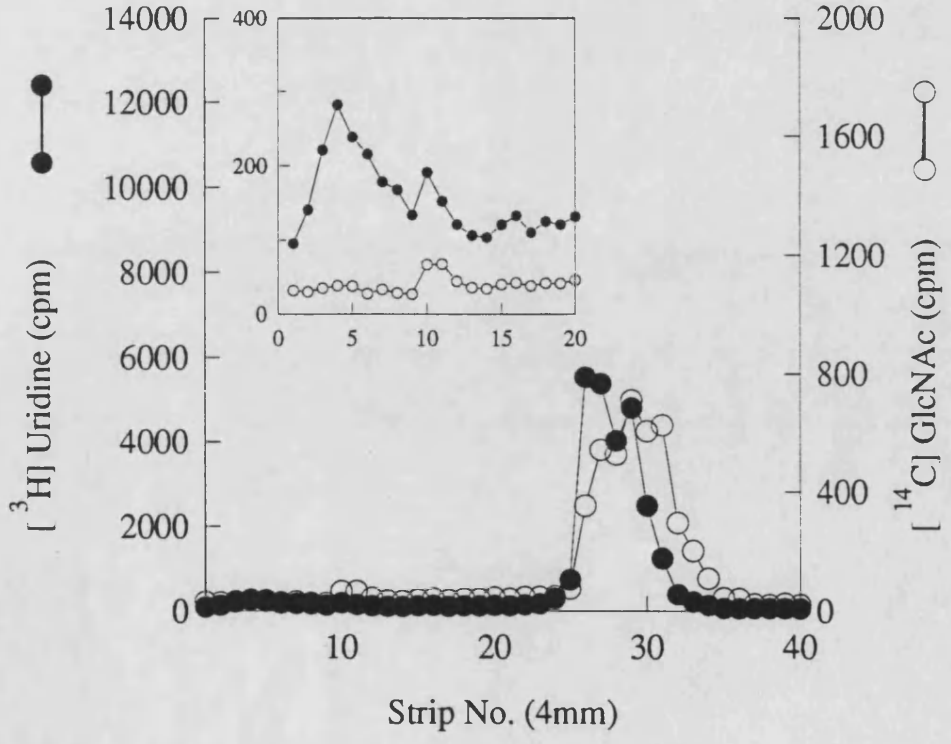
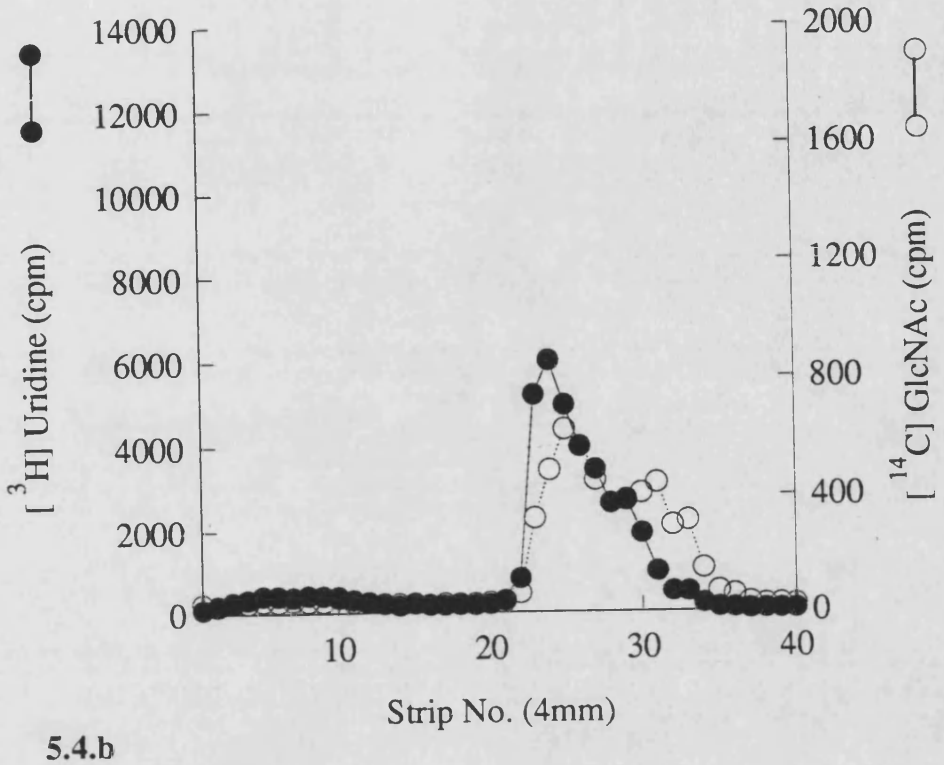

14000
12000
10000
8000
6000
4000
2000
0
400
200
0
0
5
10
15
20
2000
1600
1200
800
400
0
10
20
30
40
[ 3H] Uridine (cpm)
[ 14C] GlcNAc (cpm)
Strip No. (4mm)

**Fig. 5.4.** TLC profile of radioactivity derived from [$^{3}$H]-Uridine and [$^{14}$C]-N-acetylglucosamine incorporation into *M. sexta* epidermal tissue after 5 h incubation. **a)** Control (DMSO solvent only). **b)** Treated with flufenoxuron. Method as in Fig. 5.3.

**5.4.a**


14000
12000
10000
8000
6000
4000
2000
0
2000
1600
1200
800
400
0
10
20
30
40
[ 3H] Uridine (cpm)
[ 14C] GlcNAc (cpm)
Strip No. (4mm)

**5.4.b**

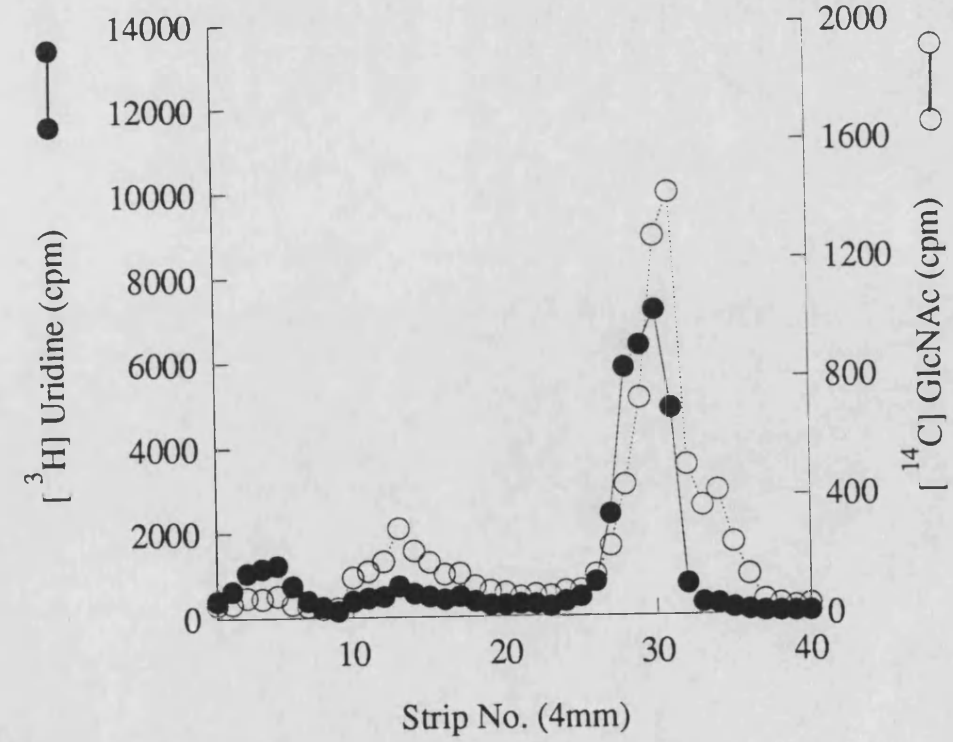

14000
12000
10000
8000
6000
4000
2000
0
2000
1600
1200
800
400
0
10
20
30
40
[ 3H] Uridine (cpm)
[ 14C] GlcNAc (cpm)
Strip No. (4mm)

**Table 5.1.**

$R_f$ values (x 100) of Bases and Mononucleotides on PEI-Cellulose TLC paper

| Nucleotide | Solvent 1[a] | 1[a]– Strip No.* | 2[b] |
|---|---|---|---|
| UMP | 32 | 13 | 62 |
| UDP | 5 | 2 | 8 |
| UTP | 2 | 1 | 1 |
| UDP-GlcNAc | 20 | 8 | 43 |
| Uridine | 85[c] | 34 | 37 |
| Uracil | 78[c] | 32 | 47 |

Table shows the $R_f$ values of bases and uridine nucleotides (10 ml per lane of 10 mM standards) developed under different solvent conditions using PEI-cellulose plastic coated TLC sheets.

[a] 1.0 N $CH_3COOH$ (20 min), 1.0 N $CH_3COOH$ - 3 M LiCl (9:1, v/v)

[b] *n*-Butanol - methanol - water - ammonia (60:20:20:1, v/v)

[c] Elongated spot

**Table 5.2**

Effect on [$^{14}$C]-GlcNAc Incorporation of aza- and fluoro- Analogues of Uracil and Uridine

| Compound | CPM incorporation into chitin Compound Dosage 0.41 μg ml$^{-1}$ | Percent[1] Control | 50 μg ml$^{-1}$ | Percent[1] Control |
|---|---|---|---|---|
| 6-Azauridine | 4100 ± 607 | 125 | 5016 ± 1555 | 118 |
| 6-Azauracil | 3731 ± 418 | 114 | 3591 ± 145 | 85 |
| 5-Fluouracil | 4608 ± 517 | 141 | 3782 ± 867 | 89 |

Table shows the incorporation by explanted proleg epidermis of [$^{14}$C]-N-acetyl-D-Glucosamine into KOH insoluble material. Means ± S.E.M. (n = 5 per point). The values presented are as a result of two separate dosage experiments. The amount of incorporation between the two doses is not significantly different using an unpaired T-test with 95% confidence intervals.

[1] relative to the amount of incorporation for control tissue (control values not illustrated).

# Chapter 6

# Synthesis of [$^{125}$I]-labelled azido-flufenoxuron and protein affinity labelling in *Manduca sexta* epidermal tissue.

## Introduction

The benzoylphenylurea insecticides inhibit the polymerisation of the chitin subunit N-acetyl-D-Glucosamine (Post *et al.*, 1974) into the chitin microfibres of the procuticle. At the biochemical level such inhibition leads to the buildup of UDP-GlcNAc in the epidermis (Hajjar and Casida, 1978; van Eck, 1979; this thesis, Chp. 5). Few studies have been done which look at the distribution of the acylurea insecticides within insect cells. Such work would require the use of radiolabelled insecticide with high specific activity. Presumably only low-specific activity labelled compounds (usually $^{14}$C-labelled) have been available. Studies using them have addressed only the issues of toxicity, metabolism etc (eg. Guyer and Neumann, 1988; Clarke and Jewess, 1990a; 1990b). Although it has been demonstrated that diflubenzuron is accumulated (to the point of crystallisation) within cells from a *Manduca sexta* cell line (Klitschka *et al.*, 1987), this work involved long-term chronic exposure of the cells to the insecticide. Little is still known about the receptor mechanisms through which these compounds may act.

Putative acylurea receptors have yet to be identified within insect cells. Identification of such receptors may finally lead to information on their density and localisation within the epidermal cell, and ultimately, answer the issue regarding their mode of action. In a study which characterised juvenile hormone (JH) receptors in *Manduca* larval epidermis, Palli *et al.* (1990) describe a

radiolabelling technique using radiolabelled analogues of JH which possessed a photolabile diazoacetate group. This group covalently attaches to any JH binding sites when irradiated with ultraviolet light. To characterise the precise biochemical properties of the acylureas such binding studies could be utilised in a similar fashion to identify potential sites relating to chitin polymerization, and by comparison, design of more selective acylurea compounds. In the present study such a technique has been used to attempt to localise and characterise an acylurea receptor for flufenoxuron. The study describes the synthesis of a radioactive, photolabile analogue of flufenoxuron, its purification and use as a biochemical tool for receptor identification. Also described is the localisation of radiolabelled flufenoxuron within cryostat tissue sections of *Manduca sexta* prolegs.

## Materials and Methods

### *Animals*

Day 0 5th stadium *Manduca sexta* were removed from the main culture room and maintained at 25°C until required (see Chp. 2., Materials and Methods)..

### *Chemicals*

The insecticides used were technical grade. WL 145364, WL 145365 and [$^{14}$C]-WL 145365 (53.2mCi mmol$^{-1}$, see Fig. 6.1 for structures) were gifts from Shell Research Ltd, Sittingbourne, Kent, UK. Na[$^{125}$I] (2300Ci mmol$^{-1}$) was from ICN Radiochemicals, Irvine, USA and N-acetyl-D-[1-$^{14}$C]-Glucosamine (GlcNAc, 58.7mCi mmol$^{-1}$) was from Amersham. All other reagents were of analytical grade from either Sigma or BDH.

*In Vitro effect of WL 145365 on Chitin Synthesis*

In order to ascertain that the [$^{125}$I]-radiolabelled analogue of flufenoxuron would have the ability to inhibit chitin synthesis it was necessary to investigate the insecticidal activity of the unlabelled analogue using the proleg preparation (see Chp. 2., Materials and Methods). Prolegs were preincubated for 15 min with WL 145365 and then for a further 60 min after the addition of 0.1μCi [$^{14}$C]-GlcNAc, in the continued presence of 145365. The doses used were 0.41 μM ($IC_{50}$ for chitin synthesis with flufenoxuron) and 10 μM (>90% inhibition with flufenoxuron). Prolegs were processed as previously described in Chp. 2.

*Preparation of Tissue Cryostat Sections*

Rear prolegs were excised and placed in phosphate buffered saline (PBS, 10 mM, pH 7.4) containing 15% sucrose for 10 min. The proleg material was then briefly dipped into O.C.T. compound (BDH) before mounting the material onto a cryostat chuck (kept at -40$^{o}$C) and then transferred to the cryostat. Once the tissue had equilibrated to the cryostat (Bright) temperature then 12 μm thick tissue sections were collected onto previously subbed slides (Poly-L-lysine, 0.1% w/v $dH_2O$). These sections were left overnight in the cryostat to ensure thorough section adhesion to the slide.

*Synthesis of [$^{125}$]I WL 145365*

Using a protocol analogous to that used for the labelling of pethidine analogues (Werner, 1989), the 4-amino analogue (WL 145364) of flufenoxuron (WL 115110) was diluted to a concentration of 1 mg ml$^{-1}$ DMSO. To 8 μl of this was added 24 μl of sodium acetate buffer (1 M, pH 5.6) and 1mCi of Na [$^{125}$I] (2300Ci mmol$^{-1}$). The iodination was initiated with 7 μl of Chloramine T (1 mg ml$^{-1}$ $dH_2O$) and left, with occasional agitation, for 6 min at room temperature. The reaction was stopped with 5 μl of $NaHSO_3$ (1 mg ml$^{-1}$ $dH_2O$) and made

basic with 5 µl 1 N NaOH. The insecticide was then extracted 3 times with 0.4 ml Ethyl Acetate "Aristar" and the pooled organic fraction dried down under $N_2$.

With the sample tube on ice the preparation was resuspended in 200 µl dioxan/water, 1:1 (vol/vol) and acidified with 30 µl 1 N HCl. This solution was treated with 30 µl $NaNO_2$ (6.9 mg $ml^{-1}$ $dH_2O$) for 15 min and then 30 µl of $NaN_3$ (6.5 mg $ml^{-1}$ $dH_2O$) for a further 20 min. Still on ice, 35 µl 1N NaOH was added and the mixture was again extracted with ethyl acetate (3 times 0.5 ml). The pooled solvent was dried with a few crystals of $Na_2CO_3$ to remove any remaining aqueous phase, transferred to another tube and dried under $N_2$ as before with the resulting 4-azido-[$^{125}$I] iodophenyl analogue of flufenoxuron ([$^{125}$I]-WL 145365) resuspended in 1 ml absolute methanol and stored at -40°C.

*HPLC purification of [$^{125}$I]-WL 145365*

4-azido-[$^{125}$I] iodophenyl analogue of flufenoxuron was purified by HPLC (Gilson). 10mM standards of analytical grade WL 143564 and WL 145365 (in 100% HiperSolv methanol) were loaded onto a C18 Spherisorb column (4.5mm ID, 5 µm particles) and their elution over a methanol gradient (70% to 100% methanol in Millipore-filtered water, see Appendix B) was detected at 254nm (Mutanen *et al.*, 1988) with a Gilson Holochrome UV detector. 10 µl of the stored, crude [$^{125}$I]-WL 145365 was loaded and run under identical conditions to the standards (1 ml $min^{-1}$ pump speed).

On initiation of the run, 30 sec (0.5 ml) fractions were collected on a fraction collector (LKB Redirac 2112) for the duration of the 50 min run cycle. These were then counted for $^{125}$I activity with an LKB 1275 Minigamma counter. HPLC analysis resolved two iodinated peaks with about 1% of the applied $^{125}$I incorporated into the larger peak. The specific radioactivity of [$^{125}$I]-WL 145365

obtained by this procedure was, assuming it to be carrier-free, was approximately 2300 Ci $mmol^{-1}$.

*Labelling of Cryostat section with [$^{125}$I]-WL 145365*

The slides were removed from the cryostat and allowed to equilibrate to room temperature in a dessicator. They were washed, once equilibrated, in PBS (10 mM, pH 7.4) for 3 times 10 min. For the radioligand incubation, each section was immersed in 16 µl test solution (5 µl HPLC purified [$^{125}$I]-WL 145365 - approx. 24 000 cpm, 1 µl DMSO and 10 µl Tris-HCl (50 mM, pH 7.4)). For non-specific binding (NSB), other sections were immersed in an identical solution but also containing 1 µl WL 145365 (analytical standard) in DMSO (1 mg $ml^{-1}$). Slides were stored in filter paper soaked trays at 4°C for 1 h. They were again washed in PBS (3 times 10 min) before the tissue was fixed with para-benzoquinone (Aldrich, 0.4% w/v in PBS) for 2 h and then allowed to dry.

*Preparation of Photomicrographs*

The slides were dipped in molten Ilford K2 emulsion (diluted 1:1 with 11.76 ml $dH_2O$ and 0.24 ml glycerol) at 43°C, drained, and placed on a sheet of cold metal for 1 h (Rogers, 1967). The slides were covered and placed in a lead-lined box at 4°C for either 2 or 6 days before being processed. After developing the slides with Ilford Phen X X-ray developer (1:1 $dH_2O$) they were taken up through an alcohol series to xylene and mounted under DPX mounting medium (BDH). Photographs were then taken using phase contrast optics on a Zeiss Photomicroscope 2 focussing on the silver grains (0.2 µm) due to the nature of the silver grains overlying the tissue.

*Preparation of larval epidermal acylurea receptors*

This method follows the technique used by Palli *et al* (1990). In more detail, all the prolegs (not just the rear ones) were removed from 10 day 0 5th

stadium larvae and homogenized on ice in buffer A (Tris-HCl, 20mM, pH 7.9. 50 mM KCL, 300 mM sucrose, and 1 mM of the inhibitors ethylenediaminetetra acetic acid (EDTA), Dithiothreitol (DTT) and phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged (1 000x g, 30 min), the supernatant stored on ice and the pellet washed twice with buffer A and once buffer B (Tris-HCl, 20 mM, pH 7.9, 5 mM magnesium acetate and the above inhibitors). The resulting nuclear pellet was highly enriched with nuclei as confirmed by phase-contrast microscopy. The nuclei were extracted with 0.5 M KCL in buffer B for 2 h on ice before being centrifuged (12 000x g, 15 min, 4°C). After centrifugation both the nuclear supernatant and the homogenization supernatant (stored on ice) were dialyzed against Poly Ethylene Glycol (PEG) crystals at 4°C before being transferred to buffer B overnight (3 changes, 4°C).

Protein concentrations of the dialyzed supernatants were determined (Bradford, 1976) using lysozyme (Sigma) as the standard.

*Photoaffinity Labelling*

All plastic surfaces were precoated with 1% (w/v) PEG (Sigma) to reduce non-specific binding. For photoaffinity labelling, 40 µl of the nuclear ( 44 mg protein $ml^{-1}$) or 200 µl of homogenate supernatant ( 40 mg protein $ml^{-1}$) was added to buffer B (total volume, 400 µl) containing the photoaffinity labelled analogue (20 µl [$^{14}$C]-WL 145365, approx 7 x $10^6$ cpm; or 10 µl [$^{125}$I]- WL 145365, approx 1.4 x $10^5$ cpm) with or without an excess of unlabelled analogue (WL 145365 in DMSO (1 mg $ml^{-1}$); 20 µl in [$^{14}$C]-analogue assay, 10 µl in [$^{125}$I]-analogue assay). Samples were incubated in plastic-well dishes (1 h 15 min, 4°C, in the dark) and the samples then irradiated for 1 min at 300 nm (Rayonet Photochemical Reactor Lamp).

*Analysis of Labelled samples by SDS/PAGE*

After irradiation, to 250 µl of each sample (8 in total) in an Eppendorf tube was added 1 ml cold acetone containing 9 mM acetic acid (Delmer *et al.*, 1987) and stored for 1 h at -20°C. The resulting protein precipitate was collected by centrifugation (10 000x g, 15min, 4°C), the acetone removed, followed by lyophilization of the precipitate. Samples were resuspended in 10 µl Tris-HCl/EDTA (10 mM/0.1 mM, pH 8.0), made up to 20 µl with 2x loading buffer and placed in a boiling water bath for 1 min to denature the proteins. Samples were loaded onto 10% acrylamide gels prepared by the procedure of Laemmli (1970). Acrylamide concentration in the stacking gel was 4.5%. Proteins were electrophoresed overnight at 50 mA, stained in Coomassie blue R250 and destained with 30% methanol/10% acetic acid. The 20 µl samples were co-run with 5 µl of molecular weight standards (Pharmacia, see Appendix C) to allow determination of the sample proteins sizes within the acrylamide gel. The gel was then incubated with Amplify (Amersham) for 30 min to increase definition of any radiolabelled proteins, transferred onto filter paper (Whatman 3MM) and subjected to fluorography using Kodak X-Omat Film at -80°C for 4 weeks.

## Results

*WL 145365 inhibition of Chitin Synthesis*

The effect of WL 145365 on chitin synthesis *in vitro* is shown in Table 6.1. From these initial experiments it was clear that WL 145365, the azido version of flufenoxuron, inhibited chitin synthesis to a similar extent. At the $IC_{50}$ concentration of flufenoxuron (0.41 µM) for cuticular synthesis, WL 145365 was almost as potent (38% inhibition) as flufenoxuron. Inhibition increased to 94.9% at 10 µM. This important result made it possible for the radiolabelling of WL 145364 to proceed having shown that the end product for the radiolabelling

reaction, although unlabelled in this experiment, could inhibit chitin synthesis *in vitro*.

*Synthesis of chemically reactive [$^{125}$I]-WL 145365*

The scheme used for the synthesis of the chemically reactive insecticide is displayed in Figure 6.1.

An aryl azide was exploited as the relevant functional group, which upon exposure to UV light, photolyses to generate a very reactive nitrene group. The radioactively labelled photoaffinity ligand [$^{125}$I]-WL 145365 was prepared from the amino-flufenoxuron starting material (WL 145364, compound 1) by a two-step reaction. This firstly involved iodination using Chloramine T and Na[$^{125}$I], and then converting to the corresponding azide compound (WL 145365) by diazotization and subsequent reaction with sodium azide. The product, compound 3, was analysed by HPLC and scintillation counting (Fig. 6.2.) and shown to have a different elution time to both native WL 145364 (Fig. 6.3.a.) and WL 145365 (Fig. 6.3.b.). The elution times of all the compounds are given in Table 6.2.

The HPLC profile of [125I]-WL 145365, compound 3, showed a characteristic double peak which was seen after each synthesis reaction. Since iodination typically leads to an increase in hydrophobicity, it was thought that the first, smaller peak (Fraction #60) was mono-iodinated and the larger peak (Fraction #62) was di-iodinated. An attempt was made to verify this hypothesis by perfoming an iodination reaction under identical conditions to those described above, except that non-radioactive NaI was used. Fraction #62 was then subjected to mass spectroscopy (performed at Shell Research Ltd, Sittingbourne). Unfortunately, no useful signal was obtained, so that identification of fraction was not possible.

*Cytochemical Labelling of [$^{125}$I]-WL 145365*

Cryostat tissue sections from *Manduca sexta* prolegs, when incubated with Fraction #62 of[$^{125}$I]-WL 145365, showed a high degree of binding. This binding was specific for the epidermal region (Fig. 6.4.a.), as judged by the number of silver grains, and was evenly dispersed throughout the epidermal material. This binding was specific since it could be reduced by the addition of 100-fold excess (as calculated from the known specific activity of Fraction #62) unlabelled WL 145365 (Fig. 6.4.b.) No difference in the level of silver granulation was seen for slides exposed for 2 days or 6 days. Therefore comparisons were made using slides from the 2 day exposures. The result showed the possible competition for receptor sites between labelled and unlabelled material.

*Photoaffinity labelling of epidermal proteins*

Coomassie staining of the SDS gel revealed distinct protein bands in the lanes containing the nuclear protein extracts. The 5 most distinct bands were 77, 51, 33.5, 28 and 13.5-kDa (Fig. 6.5). The autoradiograph of the same gel showed 4 distinct radioactive bands of 71.5, 27.5, 25.5, and 13.5-kDa (Fig. 6.6). The bottom of the autoradiogram showed heavy darkening which was presumably due to unreacted [$^{125}$I]-WL 145365 running to the bottom of the gel. Binding of the labelled insecticide to these latter proteins was only partially reduced with 1700-fold excess of unlabelled WL 145365, as judged by the NSB lane, meaning that no one protein could be singled out as a putative receptor.

Coomassie-stained SDS gel of the 1000x g supernatant (cytoplasmic proteins) showed a less distinct pattern of bands. Some bands (77, 23 kDa) appeared to be the same as those in the nuclear protein extract, but were much fainter. Much of the protein ran in an indistinct pattern of bands in the region 20-30 kDa. The autoradiogram of the SDS gel showed a different pattern of binding to the cytoplasmic extract than that seen with the nuclear extract. Most

radioactivity was bound to the 1000x g extract by 3 proteins of approximate molecular weights 66, 44 and 37 kDa. Faint bands appeared at 56, 28 and 18 kDa. Again, there was heavy darkening at the bottom of the gel, presumably due to unreacted label. Binding of the labelled insecticide was only slightly decreased by the presence of excess WL 145365.

**Discussion**

The work reported in this chapter has shown the feasibility of preparing radioiodinated azido-derivatives of flufenoxuron using a simple 2-step protocol. Unfortunately, it was not possible to confirm unequivocally the identity of the [$^{125}$I]-labelled compounds this produced, but there is good reason to believe that they correspond to mono [$^{125}$I]- and di[$^{125}$I]-labelled azido compounds (Fig. 6.1).

It would have been desirable to confirm that these iodinated azido compounds inhibit chitin synthesis. Also unfortunatley, it did not prove possible to prepare sufficient of these compounds, either radioactively or non-radioactively labelled, to test this. However, there is good reason to believe that these compounds would inhibit chitin synthesis since the non-iodinated azido derivative is a potent inhibitor of [$^{14}$C]-GlcNAc incorporation (Table 6.1) and studies of structure-activity relationships among acylurea-type compounds show that a wide range of substituents on the aniline and benzoyl component of the molecule is capable of insecticidal activity (Nakagawa *et al.*, 1984, 1987).

More work is needed to ensure that the iodinated azido-compound is indeed an inhibitor of chitin synthesis. However, the result of the *in situ* binding experiments is encouraging. When incubated with a section of integumental tissue, the labelled flufenoxuron analogue bound to epidermal cells but not to the

cuticle. Most of the bound affinity label was displaced by a 100-fold excess of unlabelled WL 145365. While this experiment must be regarded as a preliminary one, results suggest that the epidermis (but not the cuticle) contains specific binding sites for acylurea-type compounds.

If characterisation is achieved, then a detailed study of the receptor protein(s?) interaction with acylurea responsive genes is necessary for understanding receptor action at the molecular level.

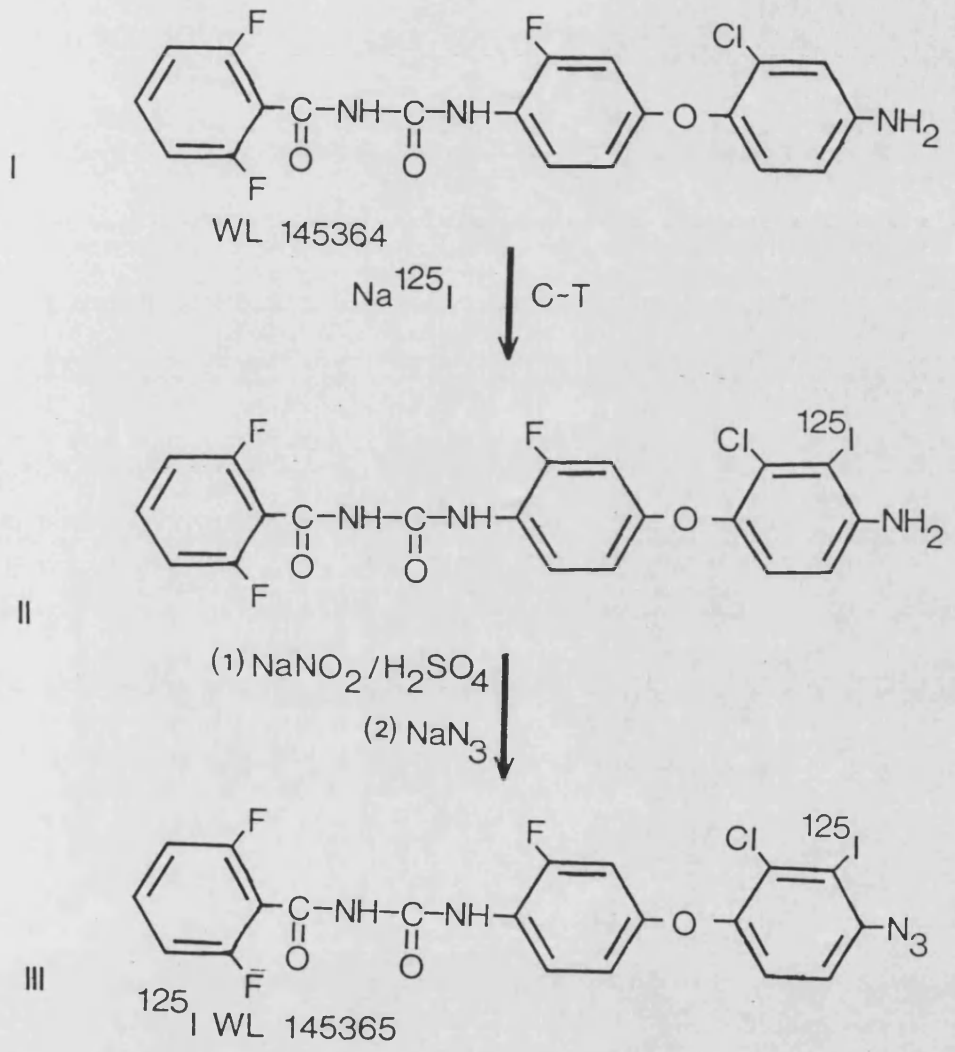



**Fig. 6.1.** Synthesis of chemically reactive flufenoxuron analogues by chloramine-T iodination followed by diazotization and subsequent reaction with sodium azide

**Fig. 6.2.** HPLC profile of [$^{125}$I]-WL 145365 when eluted over a 70-100% methanol gradient. 10 µl of the stock solution was loaded onto the column (C18 Spherisorb) and 0.5 ml samples were collected every 0·5 min for radioactive counting.

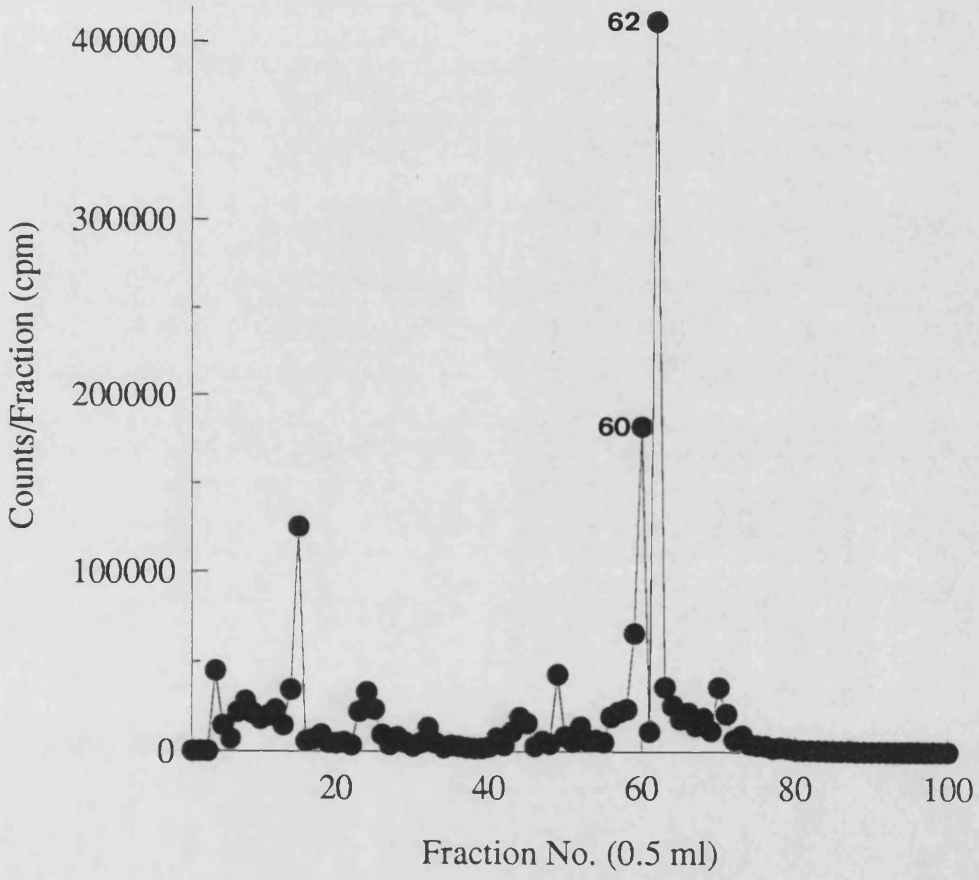
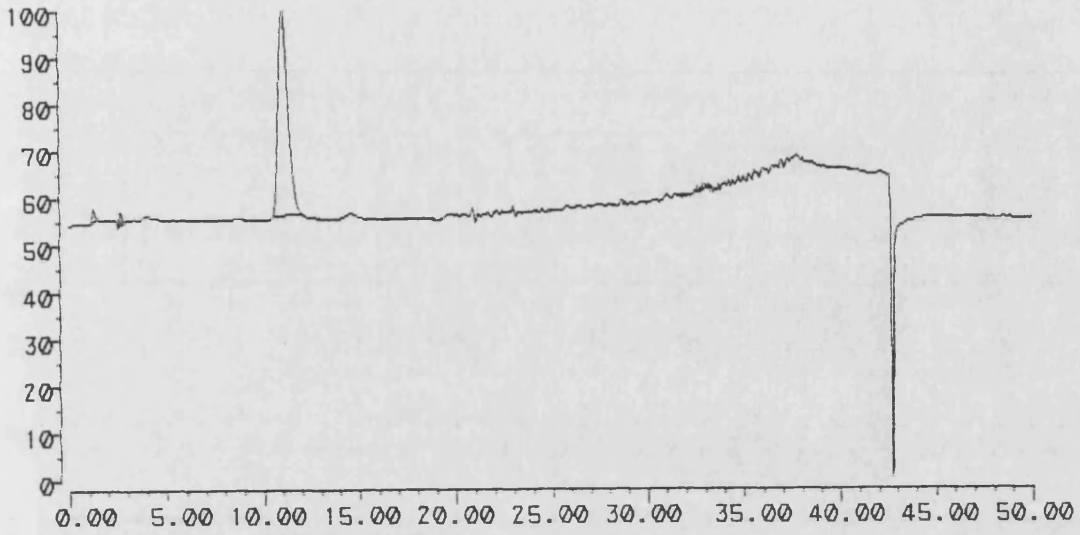

400000
300000
200000
100000
0
Counts/Fraction (cpm)
62
60
20
40
60
80
100
Fraction No. (0.5 ml)

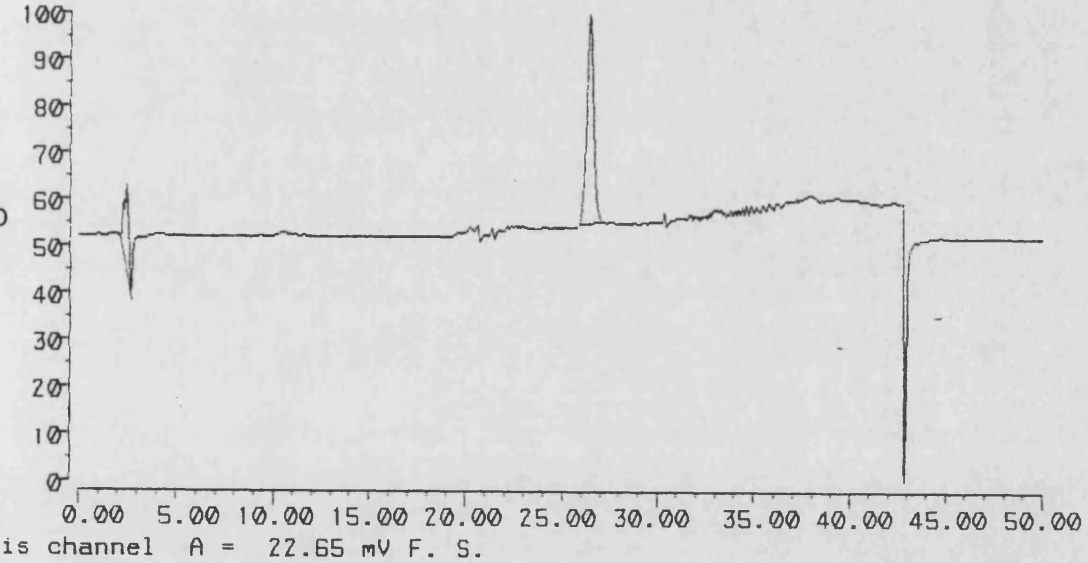
**Fig. 6.3.a.** HPLC profile of WL145364. **b**) HPLC profile of WL 145365. In each case the standards were eluted over the same gradient as for Fig. 6.2. 10 µl of the standard (1 mg $ml^{-1}$ in 100% methanol) were loaded onto the column.

a




Analysis channel A = 21.47 mV F. S.

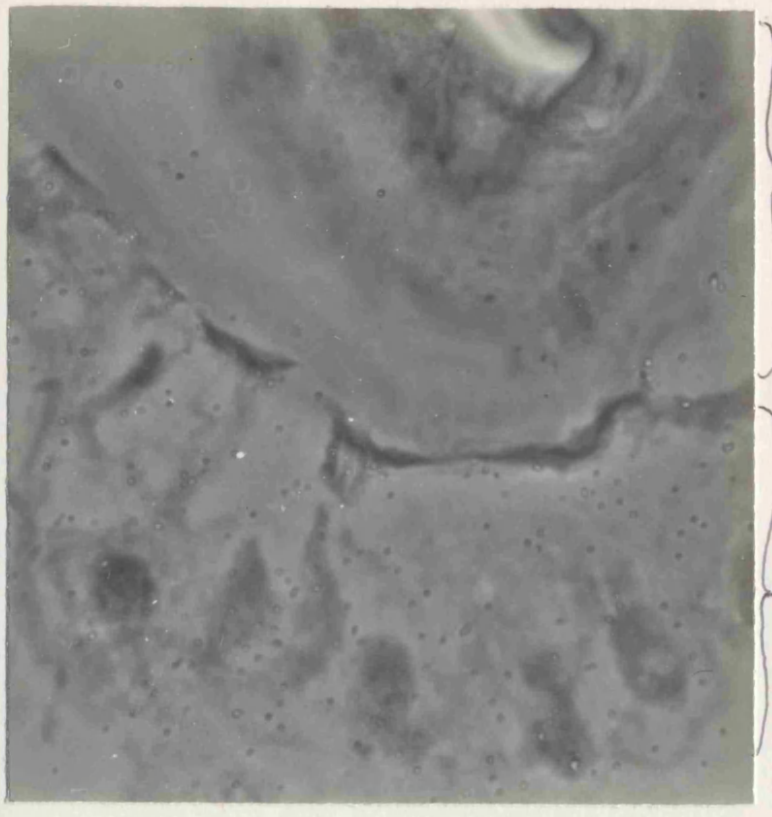
b




Analysis channel A = 22.65 mV F. S.

**Fig. 6.4.a.** *Manduca sexta* larval epidermal tissue incubated in the presence of [$^{125}$I]-WL145365. Silver granules show the distribution of the HPLC purified insecticide and the relative intensity of the insecticide localisation. **b**) As before but with 100-fold excess of the standard WL 145365. Slides were exposed for 48 hr. (x100)

a




b

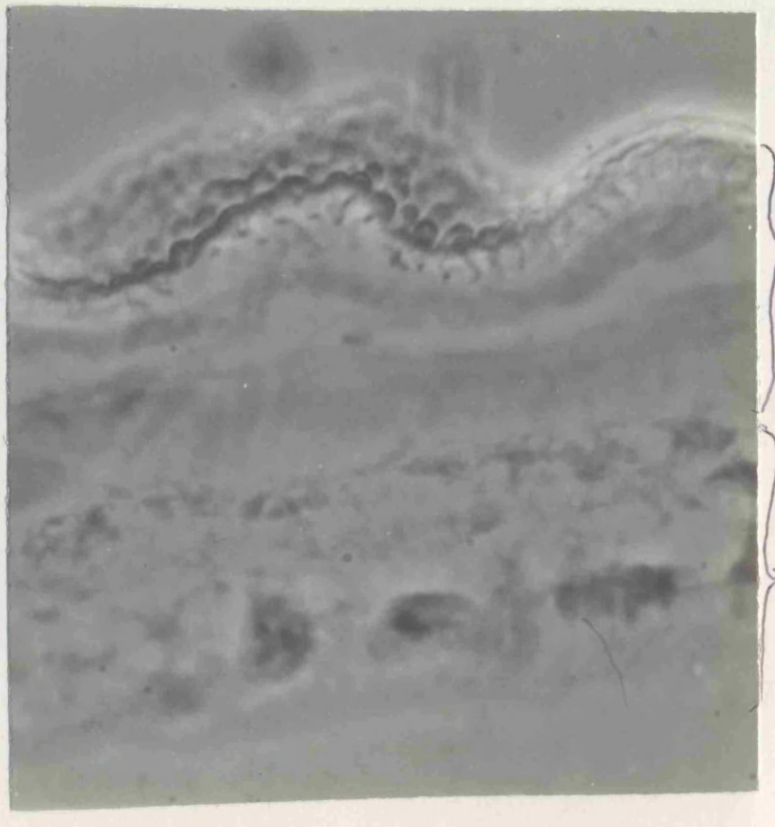


**Fig. 6.5.** Photograph of SDS gel (see Fig. 6.6.) showing the distribution of radiolabelled proteins in the nuclear (N) and the homogenate (H) extracts from *Manduca sexta* day 0 fifth stadium larvae. These were irradiated in the presence of either [$^{14}$C] or [$^{125}$I]-labelled WL 145365 alone (HOT) or with the addition of excess WL 145365 (NSB). The major protein bands are indicated with their molecular weights (kDa).

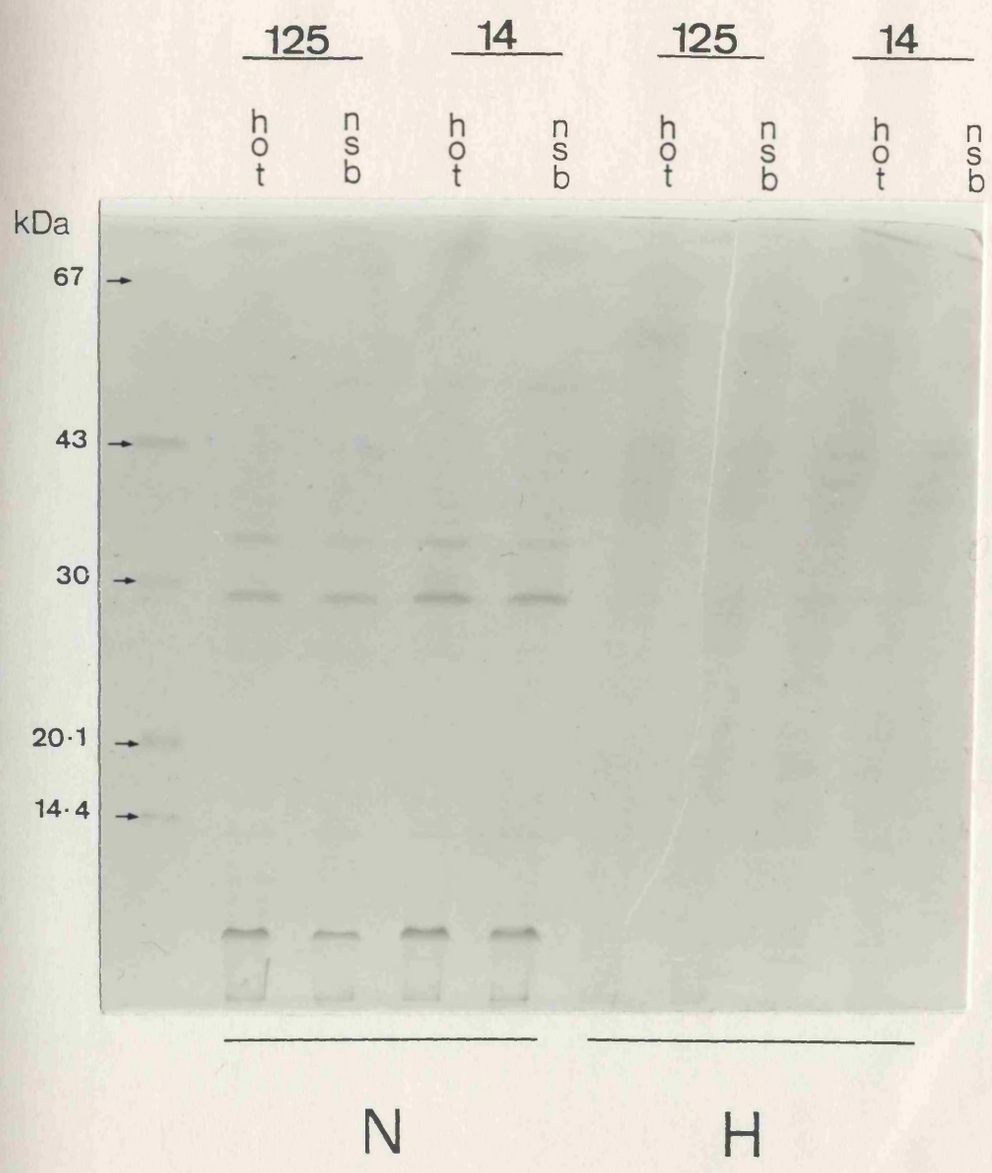

125
14
125
14
hot
nsb
hot
nsb
hot
nsb
hot
nsb
kDa
67
43
30
20·1
14·4
N
H

**Fig. 6.6.** Autoradiogram of SDS gel from Fig. 6.5. showing photolabelling of 0.5 M KCl-extractable nuclear proteins and homogenate proteins from day 0 fifth stadium *Manduca sexta*. Conditions as for Fig. 6.5. The arrows indicate the major proteins covalently modified by the photoaffinity analogue [$^{125}$I]-WL 145365.

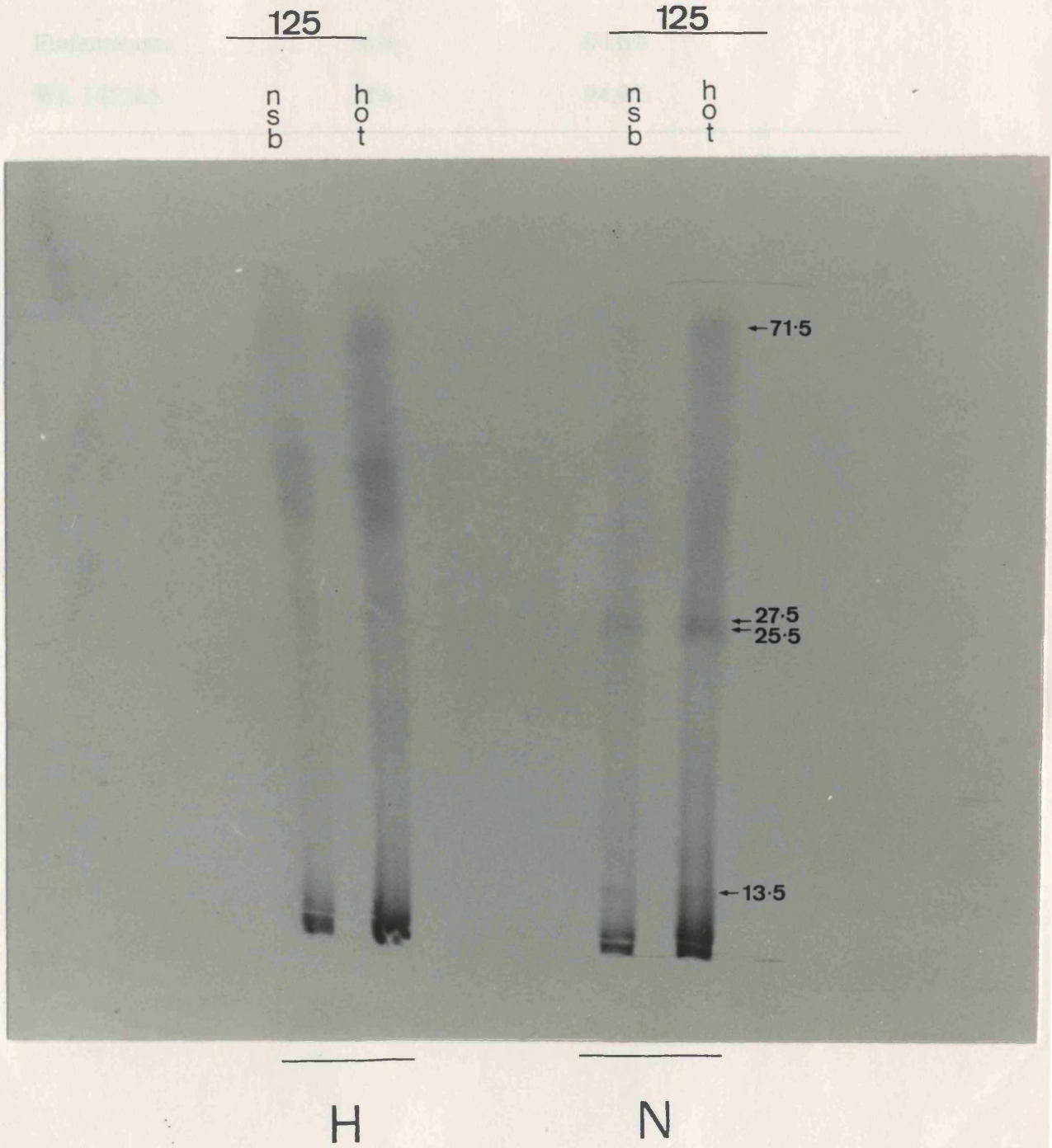

125
125
n s b
h o t
n s b
h o t
←71·5
←27·5
←25·5
←13·5
H
N

**Table 6.1.**

| Compound | Inhibition of [$^{14}$C]-GlcNAc Incorporation 0.41 μM | 10 μM |
|---|---|---|
| Flufenoxuron | 50% | 94.6% |
| WL 145365 | 38% | 94.9% |

Table shows the incorporation by explanted proleg epidermis of [14C]-N-acetly-D-glucosamine into KOH insoluble material. Means (n=5 per point).

**Table 6.2.**

HPLC Retention Times of Flufenoxuron analogues

| Compound | HPLC Elution Time (mins) | Correcting for void volume (mins) |
|---|---|---|
| WL 145364 | 11.08 | 8.40 |
| WL 154365 | 26.41 | 23.96 |
| [$^{125}$I]-WL 145365 | | |
| Fraction #60 | 30.0 | 27.5 |
| Fraction #62 | 31.0 | 28.5 |

10 µl of each of the compounds (10 mM standards) were loaded onto a C18 Spherisorb column in 100% HiperSolv methanol and eluted over a 70-100% methanol gradient. In each case both the HPLC retention time on the column and the void volume corrected time are given (approximated for the iodinated fractions).

# Chapter 7
# Summary and Overview

## Summary of Findings

A study of the development of fourth stadium *Manduca sexta* larvae when fed artificial diet containing varying doses of the acylurea, flufenoxuron, revealed that the larvae were killed in a dose-dependent manner. In some cases, larvae successfully survived the moult to the 5th stadium but these then failed to reinitiate feeding. Normally there is a tremendous burst in the growth rate where control larvae can increase from 1.3 g to >8 g in just four days in the 5th stadium. This failure to reinitiate feeding is a noted effect of acylurea action (Retnakaran *et al.*, 1985; Neumann and Guyer, 1987). If fourth stadium larvae received an injected dose of flufenoxuron instead then efficacy as a larvicide affecting ecdysis showed an increase towards the end of the fourth stadium rather than the start. This is because formation of the following fifth instar cuticle does not occur until towards the end of the fourth stadium (Jenkin and Hinton, 1966).

An *in vitro* study of the capacity for flufenoxuron to inhibit incorporation of [$^{14}$C]-GlcNAc into chitin gave a value for 50% inhibition ($IC_{50}$) as 0.41 μM. Similar toxicity values have been shown for the activity of other acylurea insecticides (Hajjar and Casida, 1979; Kitahara *et al.*, 1983).

When explanted tissue was exposed to a single dose of flufenoxuron the appearance of the apical microvilli on the epidermal cells was altered after only 1 h 15 min. However, the distribution of chitin within the cuticle was unaffected, as

determined by binding of the lectin WGA linked to gold particles. Whether this remained true for longer periods of acylurea exposure was not investigated. The only other study of this type, using flufenoxuron, showed cuticular disruption after a longer period of exposure (Lee *et al.*, 1990) in *Spodoptera littoralis*. Nevertheless, this change in microvillar regularity has been well documented (Binnington, 1985; Percy-Cunningham *et al.*, 1987). It is suggested that disruption of the cell membrane is a possible mode of action for flufenoxuron.

The response of *Manduca sexta* to flufenoxuron and a further 5 other acylureas was examined at four temperatures (20, 25, 30 and 35$^{o}$C). This response, for increasing temperature, was characterised by increased mortality and more pronounced sublethal growth inhibition. This latter observation has also been noted at a single discriminatory temperature for other recognised insect growth regulators (Kitahara *et al.*, 1983). Likewise, Lacey and Mulla (1978) showed evidence of a positive correlation of mortality with temperature for diflubenzuron against *Simulium vittatum*. This effect of temperature was also recognised for the ability of flufenoxuron, in *Manduca sexta*, to inhibit chitin synthesis where the inhibition was less effective at the lowest temperature compared to the other 3. Overall, the results suggested that the effectiveness of the acylurea insecticides is strongly influenced by temperatures likely to be encountered in the field.

Changes in the cellular levels of -uridine-derived compounds were monitored in control and flufenoxuron-treated *Manduca sexta* larvae. After 5 h, a distinct increase in the chitin precursor UDP-GlcNAc was observed over that seen in 1 h treated larvae. This is in agreement with documented effects of other benzoylphenylureas (van Eck, 1979; Gijswijt *et al.*, 1979). An increase in the level of UTP and/or UDP was also seen. It is possible that this is a cause of the accumulation of UDP-GlcNAc.

The synthesis and purification of an iodinated derivative of flufenoxuron was used as a biochemical tool for the possible identification of an acylurea-like receptor. Although no one extracted epidermal cell protein was specifically labelled, the radiolabelled bands were not all mirror images of the protein gel bands.

**Overview**

Several important conclusions can be summarized from this major study of benzoylphenylurea action, particularly that of flufenoxuron. The results demonstrate that, at the cellular level, the action of flufenoxuron is indeed very rapid. Chitin synthesis inhibition could be measured just over an hour after the start of the radiolabelled precursor experiment and that modest levels of inhibition, although perhaps not complete, could be achieved within a few minutes (undocumented). These findings were some of the first results established by early research (Post *et al.*, 1974; Deul *et al.*, 1978) using diflubenzuron, a forerunner to the more potent second generation acylureas. Longer exposure shows the evolvement of the secondary characteristics of acylurea cell toxicity, namely an increase in the epidermal content of the chitin precursor UDP-N-acetylglucosamine (Turnbull and Howells, 1982). Strangely though, this short-term inhibition of chitin synthesis could not be reflected in ultrastructural evidence for the synthesis of chitinless cuticle during treatment with flufenoxuron for same exposure length. A number of possible explanations for this discrepancy were discussed. Some progress was made towards the potential isolation of a receptor for the insecticide. Unfortunately, time constraints precluded a more thorough investigation of such a putative receptor.

This leaves many questions in the acylurea debate unanswered and also opens up a few more. Which cell mechanisms are capable of showing such a rapid response to acylurea exposure when considering that they (Acylureas) are more slower acting as insecticides than say pyrethroids? What is the role of uridine nucleotide phosphates in the cell pool and their degree of involvement within chitin synthesis? How do acylureas interact throughout this process? Is the true mechanism of action simply an alteration in the cell membrane properties? Until this study, no short-term effect of an acylurea had been examined on such a wide ranging issue. Clearly from the results presented there is a clear incentive to clarify the acylurea debate in laboratory research and within industry to produce more effective and selective compounds.

# Appendix A

## 1. Maintenance of the *Manduca* culture

Larvae were reared in a controlled environment room at 25 ± 0.5°C, 50 ± 10% relative humidity and a diapause-averting (LD 17:7h) photoperiodic regime. The lights were switched on at 7 a.m. and off at 12 p.m. The larvae were fed from hatching on artificial diet which was slightly modified from that described by Bell and Joachim (1976).

Eggs were collected daily from the adult box and allowed to hatch in large (*c.* 200 ml) plastic pots. Hatching took about 5 days and newly hatched first stadium larvae were placed on cubes of diet (*c.* 7.0 g) in small plastic pots (*c.* 30 ml) with tight fitting lids. Animals remained in these pots until ecdysis to the fifth stadium. Newly moulted 'fifths' were placed in large (*c.* 200 ml) plastic pots with more fresh diet (*c.* 25 g). Insects ceased to feed after about 5 days of the fifth stadium and began to search for a pupation site ('wandering'). Wandered animals were placed in holes (*c.* 10 cm by 2.5 cm) drilled in wooden blocks. The holes were fitted with corks and pupal development proceeded with pupae ecdysing about 10 days after wandering. They were then placed in plastic trays until they developed into pharate adults (distinguishable by their dark appearance and thinning of the cuticle). Pharate adults were placed in a tray in the adult box with a wire mesh frame provided so that newly eclosed adults could hang freely to expand their wings. The adult box (*c.* 1 $m^3$) was maintained under long day conditions and constant illumination was provided by a dim bulb (4W). A leaf mimic was used as an oviposition mimic (disposable nappy liner impregnated

with tobacco extract). Adults fed from a container of sucrose solution (10%) attached to a hollow, yellow, plastic flower.

**2. Artificial diet**

(i) Ingredients

| Premix (normal type) | g |
|---|---|
| casein (BDH) | 420 |
| wheatgerm (grocery) | 900 |
| sucrose (grocery) | 360 |
| dried bakers yeast (grocery) | 180 |
| Wesson's salts (Uniscience, ICN) | 120 |
| choline chloride (Sigma) | 12 |
| sorbic acid (Sigma) | 18 |
| cholesterol (Sigma) | 24 |

| Other ingredients | |
|---|---|
| Agar | 40 g |
| 10% formaldehyde | 8.0 ml |
| ascorbic acid | 8.0 g |
| aureomycin (Lederle) | 0.2 g |
| linseed oil (Polycell) | 4.0 ml |
| corn oil (grocery) | 4.0 ml |
| Vanderzant vitamin mixture (Uniscience, ICN) | 0.2 g |

(ii) Preparation

336 g of premix was mixed with 700 ml of boiling water in a foodmixer. 25 g of agar was mixed with 1000 ml of distilled water, cooked in a microwave oven for 15 min, and added to the premix in the blender. While this mixture was being mixed, linseed oil (4.0 ml), corn oil (4.0 ml) and 10% formaldehyde (8.0

ml) were added. The diet was allowed to cool to below 70°C and 2.0 g Vanderzant vitamin mixture, and 0.2 g aureomycin added. The mixture was mixed thoroughly. The diet was poured into a foil-lined pan,and allowed to set.

**3 Ephrussi and Beadle (1936) Insect saline**

| Chemical | g/litre |
|---|---|
| NaCl | 75 |
| KCl | 3.5 |
| $CaCl_2.2H_2O$ | 2.1 |

composition of final saline

| | mM |
|---|---|
| $Na^+$ | 128 |
| $K^+$ | 5 |
| $Ca_2^+$ | 2 |
| $Cl^-$ | 135 |

A 10x stock solution was prepared which was diluted for use as necessary.

**4. *Manduca* Saline**

| 10x Salts | 100x Buffer |
|---|---|
| NaCl 2.34 g | $Na_2HPO_4$ 23.4 g/litre |
| KCl 29.84 g | $NaH_2PO_4$ 21.3 g/litre |
| $MgCl_2.6H_2O$ 34.3 ml | Mix the above two solutions to pH 6.9 |
| $CaCl_2$ 3.33 g | |
| (or $CaCl_2.2H_2O$) 4.41 g | |

Make up to 1 litre with distilled water

100 ml 10x Salt solution

10 ml 100x Buffer solution

66 g sucrose

Make saline up to 0.5 l by adding distilled water and dissolve all the sucrose. Bring the volume up to 1 litre adjusting the pH to 6.5 with either 0.1 M HCl or KOH.

**5. Grace's Insect Tissue Culture Medium**

| Chemical | mg/litre | Chemical | mg/litre |
|---|---|---|---|
| L-Isoleucine | 50 | L-Phenylalanine | 150 |
| L-Tryptophan | 100 | L-Leucine | 75 |
| L-Histidine HCl | 3378 | L-Methionine | 50 |
| L-Valine | 100 | L-Arginine HCL | 700 |
| L-Lysine HCL | 625 | L-Threonine | 175 |
| L-Asparagine $H_2O$ | 397.7 | L-Proline | 350 |
| L-Glutamine | 600 | DL-Serine | 1100 |
| Glycine | 650 | L-Alanine | 225 |
| β-Alanine | 200 | L-Cystine | 22.69 |
| L-Tyrosine | 62.15 | L-Glutamic Acid | 600 |
| L-Aspartic Acid | 350 | D-Sucrose | 26680 |
| D-Fructose | 400 | D-Glucose | 700 |
| L Malic Acid | 670 | α-Ketoglutaric Acid | 370 |
| D-Succinic Acid | 60 | Fumaric Acid | 55 |
| p-Aminobenzoic Acid | 0.02 | Folic Acid | 0.02 |
| Riboflavin | 0.02 | Biotin | 0.01 |
| Thiamin HCl | 0.02 | D-Calcium pantothenate | 0.02 |
| Pyridoxine HCL | 0.02 | Nicotinic Acid | 0.02 |
| i-Inositol | 0.02 | Choline Chloride | 0.20 |
| $NaH_2PO_4.2H_2O$ | 1140 | $CaCl_2.2H_2O$ | 1325 |
| $MgCl_2.2H_2O$ | 2280 | $MgSO_4.7H_2O$ | 2780 |
| KCl | 2240 | $NaHCO_3$ | 350 |

## Appendix B

Gilson HPLC Gradient profile

| TIME (MINS) | %A | %B |
|---|---|---|
| 0.00 | 100 | 0.0 |
| 16.00 | 100 | 0.0 |
| 35.00 | 0.0 | 100 |
| 40.00 | 0.0 | 100 |
| 40.01 | 100 | 0.0 |
| 50.00 | 100 | 0.0 |

Solvent for pump A is 70% 'HiperSolv' Methanol

Solvent for pump B is 100% 'HiperSolv' Methanol

Flow Rate is 1ml $min^{-1}$

HPLC Column is a Spherisorb C18 column (ID 4.5mm, 5 μm particles)

## Appendix C

Size of Molecular Weight Markers used in SDS/PAGE gel

| Protein | Molecular Weight (kDa) |
|---|---|
| Bovine Serum Albumen | 67 |
| Ovalbumin | 43 |
| Carbonic Anhydrase | 30 |
| Soybean Trypsin Inhibitor | 20.1 |
| α-Lactalbumin | 14.4 |

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# Temperature Effects on the Action of Acylurea Insecticides against Tobacco Hornworm (*Manduca sexta*) Larvae

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*ABSTRACT*

*The toxic effects of six acylurea insecticides on larvae of the tobacco hornworm were investigated at each of four environmental temperatures (20, 25, 30 and 35°C). This spans the range of temperatures which the insects can tolerate. For all the acylureas tested, mortality increased with temperature when either newly hatched or fourth-instar larvae were given insecticide in their food. Sub-lethal growth inhibition also became more pronounced at progressively higher environmental temperatures. This temperature dependence of acylurea action was not due to altered uptake of the insecticide, since there was no significant variation with temperature in the amount of [$^{14}$C]flufenoxuron taken up by fifth-instar larvae when given a single meal containing labelled insecticide. Additionally, mortality of fourth-instar larvae given a single intra-haemocoelic injection of flufenoxuron was significantly greater at higher temperatures, implying that temperature affects a process that occurs after insecticide uptake. The intrinsic ability of acylureas to inhibit chitin synthesis is temperature-sensitive, since flufenoxuron inhibited the incorporation of* [$^{14}$C]N-*acetylglucosamine into chitin by proleg epidermis* in vitro *significantly less well at 20°C than at the higher temperatures tested. However, there was no significant variation between the effectiveness of in-vitro chitin synthesis inhibition at* 25, 30 *and* 35°C. *These data show that the effectiveness of acylurea insecticides is subject to strong temperature effects in the range of temperatures likely to be experienced in the field.*



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## 1 INTRODUCTION

Acylurea (benzoylphenylurea) insecticides are agents with an insect growth regulator (IGR)-like action that results in delayed mortality of the treated insects, which usually die at the time of the next moult.[1,2] The cause of death is usually the rupture of the new cuticle during ecdysis, although in some cases the affected insects moult apparently successfully, but then fail to thrive and subsequently die.[3] Sub-lethal effects can include a reduced rate of feeding and growth.[4–6]

Since the introduction of the first commercially useful acylurea agent, diflubenzuron,[7] a number of other promising compounds have been developed. All appear to share the same mode of action, which is the inhibition of chitin synthesis.[8] Failure to deposit chitin microfibrils in newly deposited cuticle renders it weak and ductile, especially at the time of the moult, accounting for the observed symptoms.[9,10]

Little is known about the effect of environmental temperature on acylurea action. It was reasoned that the delay between exposure to the agent and subsequent death might cause the efficacy of these insecticides to be temperature dependent. The problem has been investigated using a range of acylurea compounds against a model Lepidopteran insect, the tobacco hornworm, *Manduca sexta*.

## 2 MATERIALS AND METHODS

### 2.1 Insects

Tobacco hornworms, *Manduca sexta* (L) (Lepidoptera: Sphingidae), were reared on artificial diet according to standard methods.[11] The main culture was kept at 25°C under a long-day photoperiodic regime (L:D, 17:7). Larvae were transferred to the test temperatures (20, 25, 30 and 35°C) immediately before testing as required. These temperatures were maintained in cooled incubators, each similarly fitted with lighting in phase with the original photoperiod of the stock-rearing room.

### 2.2 Chemicals

Insecticides were technical grade samples. Chlorfluazuron was a gift from Dr R. Neumann, CIBA GEIGY AG, Basel, Switzerland; hexafluron was from Mr P. K. Leonard, Dow Chemical Co., Letcombe Regis, UK; teflubenzuron was from Dr W. Ost, Celamerck GmbH, Ingelheim am Rhein, FRG (now Shell Forschung); diflubenzuron, triflumuron and flufenoxuron were obtained from Shell Research Ltd, Sittingbourne, UK. [$^{14}$C]flufenoxuron (0·11 $\mu$Ci mmol$^{-1}$) was synthesised at Shell Research, while *N*-acetyl-D-[1-$^{14}$C]glucosamine (58·7 mCi mmol$^{-1}$) was from Amersham. All other chemicals were of analytical grade from either Sigma or BDH.

### 2.3 Insecticide treatments

Where insecticides were incorporated into artificial diet this was done by mixing the appropriate amount of a 1 mg ml$^{-1}$ stock solution in acetone into the diet

mix after it had cooled to 70°C. Made in this way, the content of acetone in the diet was never more than 0·1%. Controls were given a diet containing the same amount of acetone as the experimental diet. For hatchlings, mortality and weight gain were assessed after 7 days of continuous exposure at the test temperature to the insecticide-treated diet. For fourth-stage larvae, insects were exposed at the test temperature to insecticide-treated diet only during that stage, and then transferred to untreated diet (maintaining the test temperature until assessment). In this case, mortality was assessed up to and including pupal ecdysis.

For injection, the stock solution of insecticide in acetone was rapidly mixed with an insect saline solution[12] to form a suspension. This was injected into the abdomen of the water-anaesthetised insect from an SGE microsyringe equipped with a 28 swg needle. Controls received acetone/saline without insecticide. After injection the insects were briefly dipped in 70% ethanol to prevent infection. Test insects were fourth stage larvae less than 24 h after ecdysis. Mortality was assessed up to the onset of wandering.

### 2.4 Uptake of [$^{14}$C]flufenoxuron

Day 0 fifth-instar larvae were fed at 20, 25, 30 and 35°C on a treated diet containing 0·03 mg kg$^{-1}$ [$^{14}$C]flufenoxuron for 24 h. At the end of this period, the larvae were killed by freezing and dissected into carcass and gut, and faeces were collected. The samples were lyophilised prior to combustion in a sample oxidiser (Packard); radioactivity was measured as [$^{14}$C]carbon dioxide. The amounts of radioactivity in the samples were expressed as a percentage of the total radioactivity (carcass, gut and faeces) recovered from each insect's carcass.

### 2.5 Chitin synthesis *in vitro*

Day 0 fifth-stage gate 2 larvae[13] were anaesthetised in carbon dioxide and surface-sterilised by swabbing with ethanol. The rear prolegs were removed and transferred to *Manduca* saline. Prolegs were first preincubated (15 min) at 20, 25, 30 or 35°C in 100 µl *Manduca* saline containing (control) 5 g litre$^{-1}$ dimethylsulphoxide (DMSO), or (experimental) 41 µM flufenoxuron in 5 g litre$^{-1}$ DMSO. Subsequently, the prolegs were transferred to identical solutions containing 17 µM[$^{14}$C]*N*-acetylglucosamine (0·1 µCi) for exactly 1 h. The incubation was stopped by transferring the prolegs to aqueous potassium hydroxide (500 g litre$^{-1}$; 0·3 ml) at 100°C for 30 min.[14] Alkali-insoluble material was washed twice in distilled water and counted directly in Optiphase Safe scintillant (LKB; 10 ml) in an LKB 1217 Rackbeta scintillation spectrometer. 87% of the radioactivity incorporated into KOH-insoluble material in this assay is chitin, as determined by solubilisation with fungal chitinase (D. R. Chandler, unpublished).

### 2.6 Statistics

Treatments at different temperatures were compared using a one-way analysis of variance, deriving 95% confidence limits from pooled standard deviation using MINITAB. Values for $LC_{50}$ were estimated by probit analysis using GENSTAT.

## 3 RESULTS

### 3.1 Temperature and acylurea action on *Manduca* larvae

When newly hatched first stage *Manduca* larvae were exposed to discriminating doses of six different acylurea insecticides, it was found that in every case mortality increased progressively with temperature in the range 20°C to 35°C (Fig. 1). The concentrations of the different insecticides used differed because some are more toxic to *Manduca* than others, but the actions of all the insecticides were affected by temperature in the same way.

It was observed for all the insecticides tested that the treated insects grew more slowly (gained less weight) than did controls, even where the treatment did not

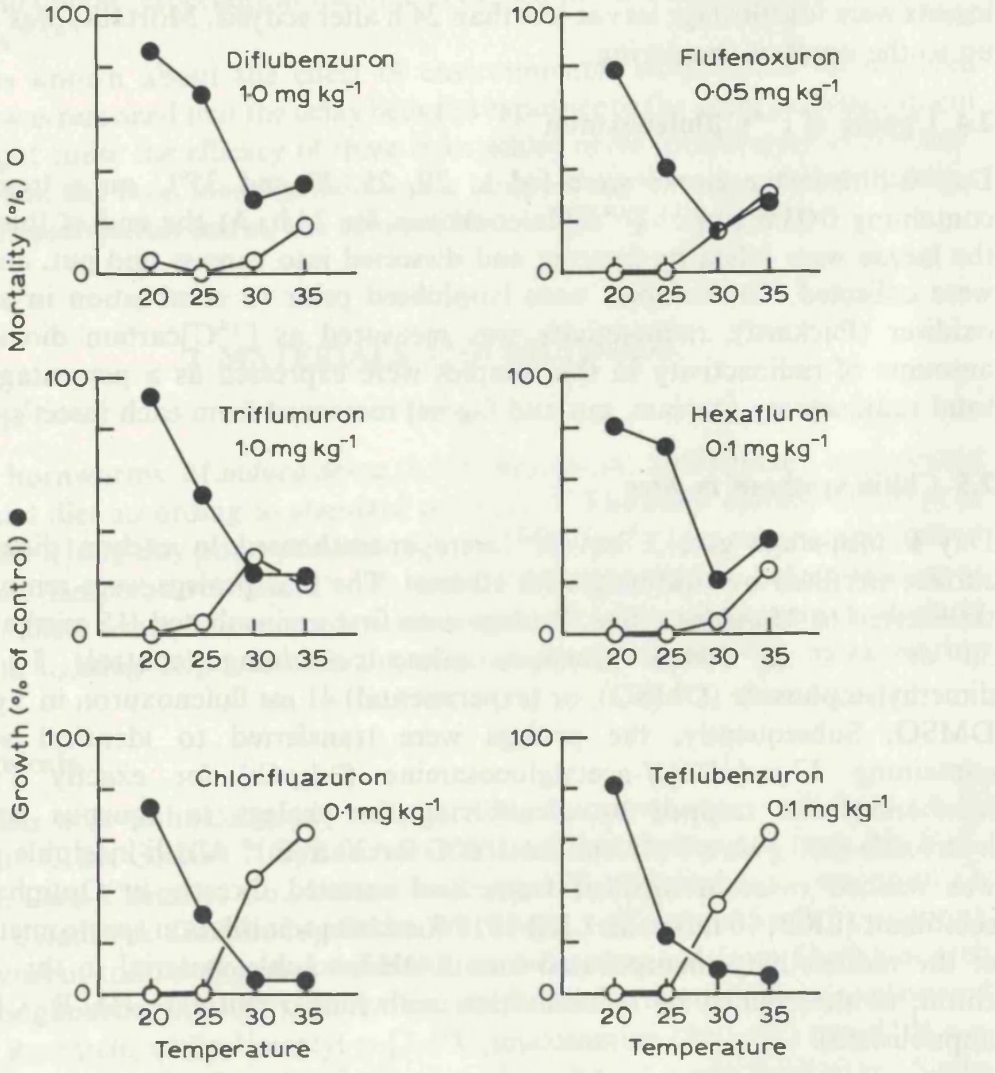


**Fig. 1.** The effects of temperature on lethal and sub-lethal effects of six acylurea insecticides on *M. sexta* larvae. Insecticides were incorporated into artificial diet at the concentrations shown. Newly hatched larvae were kept on a treated or untreated diet at the indicated temperatures. Mortality and growth in weight were assessed after 7 days. Mortality (○) is expressed as a percentage of the total number of animals (20) in each group. Growth (●) is expressed as a percentage of the control value at the same temperature.

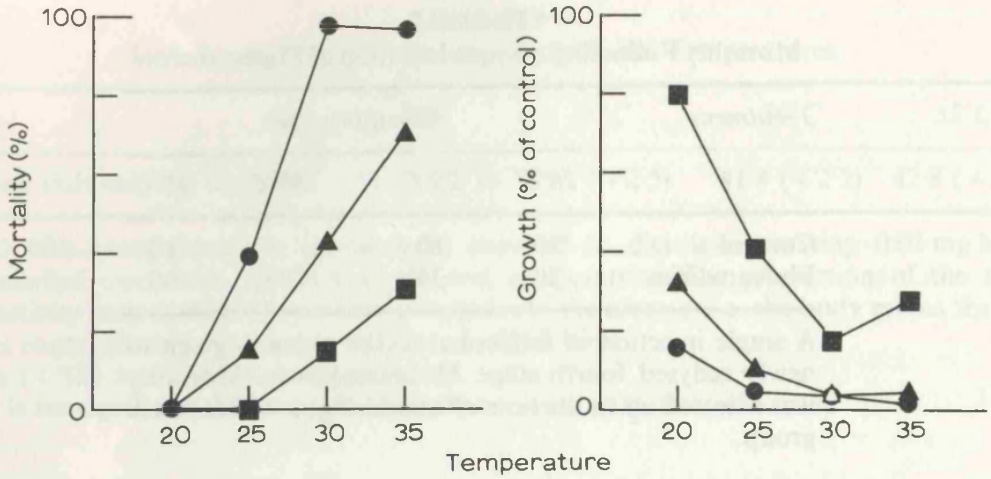


**Fig. 2.** The effect of temperature on lethal and sub-lethal effects of three different concentrations of flufenoxuron on *M. sexta* larvae (●) 0·2; (▲) 0·1, (■) 0·05 mg $kg^{-1}$. Other details as for Fig. 1.

**TABLE 1**
Toxicity of Flufenoxuron for Fourth Stage *M. sexta* Larvae (mg $kg^{-1}$)

| | *20°C* | *25°C* | *30°C* | *35°C* |
|---|---|---|---|---|
| $LC_{50}$ (±95% c.i.) | 0·041 (±0·005) | 0·039 (±0·006) | 0·021 (±0·005) | 0·020 (±0·005) |

Flufenoxuron given in the diet during the fourth stage only. Mortality is assessed up to the time of wandering. $LC_{50}$ estimated from probit analysis using data from 9 different concentrations of insecticide between 0·005 and 0·20 mg $kg^{-1}$ ($n=10$ for each concentration).

produce any mortality (Fig. 1). This sub-lethal effect, like that on mortality, was also positively correlated with temperature. Weight gain was less as a percentage of control at higher temperatures than at lower temperature.

The situation was examined in more detail for flufenoxuron, where three different concentrations were tested. The conclusion was the same: that at any dose, both mortality and growth inhibition increased progressively with temperature (Fig. 2).

This effect of temperature was not confined to treatments of first-stage larvae. When fourth-stage larvae were given various concentrations of flufenoxuron in their diet, it was again found that mortality was greater at higher temperatures. Estimated $LC_{50}$values were obtained for the insecticide's action at each temperature (Table 1). Flufenoxuron was significantly more toxic at 30 and 35°C than at 20 and 25°C.

### 3.2 Temperature and acylurea uptake

The effect of temperature on acylurea toxicity might have arisen from different rates of uptake of the insecticide. This problem was investigated in three ways.

**TABLE 2**
Mortality Following a Single Injection of Flufenoxuron

| *Treatment* | *Mortality (%)* | | | |
|---|---|---|---|---|
| | *20°C* | *25°C* | *30°C* | *35°C* |
| Control | 10 | 10 | 5 | 10 |
| Flufenoxuron | 20 | 35 | 95 | 90 |

A single injection of flufenoxuron (80 ng) was given to newly ecdysed fourth-stage *M. sexta* larvae. Mortality was assessed up to the time of wandering; $n = 20$ for each group.

**TABLE 3**
Intake of Food and Insecticide at Different Temperatures

| | *20°C* | *25°C* | *30°C* | *35°C* |
|---|---|---|---|---|
| Diet eaten per day (g)[a] | 0·58 (±0·02) | 0·79 (±0·03) | 0·90 (±0·02) | 0·88 (±0·0) |
| Diet eaten in fourth stage (g)[a] | 2·60 (±0·07) | 2·37 (±0·08) | 2·26 (±0·09) | 2·20 (±0·0) |
| Estimated $LD_{50}$ flufenoxuron intake per day (ng $d^{-1}$)[b] | 21 | 27 | 17 | 16 |
| Estimated $LD_{50}$ flufenoxuron intake during 5th stage (ng)[b] | 94 | 82 | 42 | 39 |

[a] Means (±SE) ($n = 10$).
[b] Calculated from mean values of diet eaten (this table) and $LC_{50}$ (Table 1) and thus no statistical estimate of their accuracy can be made.

First, single injections of a discriminating dose of flufenoxuron were administered to newly ecdysed fourth-stage larvae. Since this route of administration bypasses absorption from the gut, this experiment ought to show whether the effect of temperature is on insecticide uptake. As is shown in Table 2, there was still a marked dependence of mortality on temperature, implying that temperature effects on flufenoxuron uptake are, at most, minor.

Second, the amounts of food eaten by fourth-stage larvae were measured at each experimental temperature during the period in which they were exposed to insecticide, and thus the dose of insecticide to which the insects were effectively exposed was computed. Table 3 shows that although the insects reared at higher temperatures ate more rapidly, they actually ate less food in total because the duration of the fourth stage was shorter at higher temperatures. Calculated values of insecticide intake from the $LC_{50}$ concentration in the diet showed that the amount of flufenoxuron required to cause 50% mortality was less at higher temperatures than at low temperatures, whether this is computed in terms of intake per day or total intake.

In a third approach, the uptake of radiolabelled insecticide from the diet over a 24-h period was measured. It was necessary to use fifth-instar larvae (which were larger and ate more food) for this experiment because the specific activity of

**TABLE 4**
Uptake of [$^{14}$C]Flufenoxuron at Different Temperatures

| | *20°C* | *25°C* | *30°C* | *35°C* |
|---|---|---|---|---|
| % Total radioactivity absorbed | 51·6 (+5·5) | 43·2 (+2·5) | 41·8 (+2·2) | 42·8 (+2·6) |

Day 0 fifth-instar *Manduca* larvae were exposed to diet incorporating 0·03 mg kg$^{-1}$ radiolabelled insecticide for 24 h at the test temperature. The proportion of the total radioactivity consumed that was incorporated into the carcass (i.e. the body minus the gut and its contents) was determined at the end of this time.
Means (+SE) ($n=5$ for each temperature).
There is no significant difference between treatments (ANOVA, $F=1·72$).

the available labelled insecticide (and therefore the amount of radioactivity accumulated) was low. The amounts of [$^{14}$C]flufenoxuron taken up into the carcass or the gut by fifth-instar larvae did not differ significantly ($P>0·05$) between the four environmental temperatures when expressed as a percentage of the total amount of label eaten (Table 4). Thus the efficiency of insecticide uptake was not affected by temperature. Since the proportion of radioactivity present in the faeces did not vary between the different temperature regimes, it can be concluded that the extent of excretion of absorbed insecticide was also unaffected by temperature.

### 3.3 Temperature and acylurea inhibition of chitin synthesis

The effect of temperature on the toxicity of acylureas might be a consequence of a change in the intrinsic ability of the insecticides to interact with their primary target in the insect; the chitin synthetic processes of epidermal cells.

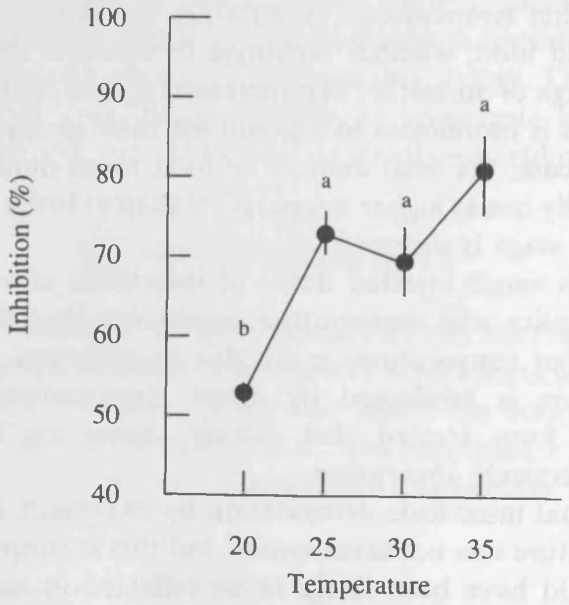


**Fig. 3.** The effect of temperature on the inhibition by flufenoxuron of chitin synthesis *in vitro*. Chitin synthesis was measured as incorporation by explanted proleg epidermis of [$^{14}$C]*N*-acetylglucosamine into KOH-insoluble material. Means (±SE) ($n=4$ per point). Significantly different values (ANOVA, $P<0·05$) are indicated by different letters.

The rate of chitin synthesis was measured *in vitro* as the rate of incorporation of [$^{14}$C]*N*-acetylglucosamine into KOH-insoluble material using explanted integument from the abdominal prolegs of day 0 fifth-stage larvae. The effect of acute exposure to flufenoxuron on chitin synthesis was tested by exposure to the insecticide during a 15 min preincubation period before the addition of the labelled precursor to the medium, using a concentration previously found to give submaximal inhibition of synthesis.

Under these conditions, flufenoxuron inhibited chitin synthesis significantly less well at 20°C than at the other temperatures tested (Fig. 3). However, there was no significant variation between the effectiveness of in-vitro chitin synthesis inhibition at 25, 30 and 35°C.

## 4 DISCUSSION

This study has shown that the action of a number of acylurea insecticides against the tobacco hornworm, *Manduca sexta*, is strongly affected by temperature. Mortality and sub-lethal inhibition of growth are both enhanced with increasing environmental temperature. The case of flufenoxuron has been investigated the most thoroughly, but the findings indicate that all acylureas tested are affected by temperature in the same way.

The effects are substantial. In experiments with single discriminating concentrations of insecticide given in their food to newly hatched larvae, the mortality experienced in the range 20°C to 35°C could vary from zero to 95% (e.g. 0·2 mg kg$^{-1}$ flufenoxuron). The estimated $LC_{50}$ for flufenoxuron against fourth-instar larvae varied by a factor of two.

The increased mortality and sub-lethal growth inhibition seen in insects exposed to acylureas at higher temperatures is certainly not due to an increased intake of insecticide-treated food, whether reckoned in terms of the intake per day or over the feeding stage of an instar. The increased rate of food intake of insects at higher temperatures is insufficient to account for their increased sensitivity to the insecticide. In any case, the total amount of food eaten during the feeding stage of an instar is actually less at higher temperature than at lower ones; this is because the duration of the stage is shorter.[15]

Experiments with single injected doses of insecticide show a similar positive correlation of mortality with temperature, suggesting that the increased efficacy of acylureas at higher temperatures is not due to enhanced absorption from the gut. This conclusion is reinforced by direct measurement of the uptake of [$^{14}$C]flufenoxuron from treated diet, which shows no significant effect of temperature on insecticide absorption.

The possibility that insecticide detoxication by enzymatic modification may be affected by temperature was not investigated, but this is considered to be unlikely, since: (a) this would have been likely to be reflected in increased excretion of radioactively labelled material (this did not occur); and (b) temperature affected in essentially the same way the efficacy of both diflubenzuron, which is subject to extensive metabolic detoxication by treated insects, and chlorfluazuron, which is

metabolised only rather slowly.[3] Nevertheless, this possibility cannot be entirely excluded at present.

The intrinsic ability of flufenoxuron to inhibit chitin synthesis, thought to be the principal target site of acylurea insecticides,[8] was clearly affected by temperature. Inhibition *in vitro* was significantly less at 20°C than at the three higher temperatures. However, there was no significant variation in inhibition between 25 and 35°C, suggesting that temperature effects on target site interactions are insufficient to account for all of the temperature dependence that was observed in experiments *in vivo*.

The remaining possibility is that insects kept at higher temperatures are intrinsically more susceptible to the inhibition of chitin synthesis, perhaps because their cuticle is qualitatively or quantitatively different from that of insects kept at lower temperature, or even because they are in some other way less 'fit',[15] and therefore less able to withstand the weakening of their cuticle. At present there is no information as to the nature of any such change in susceptibility.

The only other study that reports the effect of temperature on acylurea action concerns the action of diflubenzuron against *Simulium vittatum*, where a similar positive correlation of mortality with temperature was noted.[16] The present study raises the possibility that such a relation may be general.

Temperature effects on insecticide efficacy are complex, and many interacting variables may be involved.[17] The unambiguous relation between temperature and acylurea toxicity to *M. sexta* exposed in this study seems worthy of note. The increased mortality due to acylurea treatment at high temperature was consistently seen, regardless of larval stage or method of administration.

These effects occur within the range of temperatures that the insect is able to tolerate during larval life,[15] and to which the insects are likely to be exposed in the field.[18] It is not clear whether the temperature dependence of acylurea action is likely to be of significance in practical pest control; under field conditions, environmental temperature is almost certain to fluctuate considerably during each 24-h period, and perhaps to a lesser extent from day to day. The present findings suggest that it might at least be worthwhile to investigate the relationship of temperature to acylurea efficacy under real or simulated field conditions.

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